ROBUST SUMMARY
OF INFORMATION ON

Substance Group

Distillate Fuels

Summary prepared by American Petroleum Institute

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NB. Reliability of data included in this summary has been assessed using the approach described by Klimisch et al.

Klimisch, H. J., Andreae, M. and Tillman, U, (1997)
A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data.
Regulatory Toxicology and Pharmacology 25, 1-5.

1.1.1 GENERAL SUBSTANCE INFORMATION

Substance type : Petroleum product
Physical status : Liquid

Remark : The principal distillate fuels are:
Automotive fuels for diesel engines
- Automotive gas oil (AGO)
- Automotive diesel fuel (DERV)
- Diesel fuel No. 2
- Railroad engine gas oil

Heating oils
- Domestic heating oil
- Industrial heating oil
- Industrial gas oil
- No. 2 fuel oil

Marine fuel
- Distillate marine diesel fuel

The distillate fuel oils listed above are composed of gas oil streams that have been blended to meet specific performance criteria.
The streams that are used may be either straight run or cracked and/or hydrotreated gas oils.

Typical concentration ranges of the refinery stream components for the various fuel types have been published by CONCAWE (1995):

Process stream	**Automotive gas oil**	**Heating oil**	**Distillate marine fuel**
	Concentration range (% v/v)		
Straight-run atmospheric gas oil			
-light	40-100	40-100	40-100
-heavy	0-30	0-40	0-50
Vacuum gas oil	0-10	0-15	0-20
Thermally cracked gas oil	0-20	0-30	0-30
Light catalytically cracked gas oil	0-25	0-35	0-40

The physical chemical characteristics and other descriptors for the samples on which toxicological studies have been conducted are summarized below.

DIESEL FUELS
API Samples:

	API sample No. 79-6	**No.2 DA**
Boiling range (ASTM D-56)	367-675° F	372-656° F
Flash point (ASTM D-56)	142° F	136° F
Wt. Sulphur %	0.19	0.1
Viscosity @ 100° F (ASTM D-445)	2.17 cSt	2.4 cSt

Acid No. (ASTM D-974)	0.0	
Bromine No. (ASTM D-1159)	1.42	0.6
Benzene	80 ppm, v/v	47 ppm, v/v
Hydrocarbon types identified by mass spectrometry were:		
Paraffins	41.7 Wt. %	42.7 Wt. %
Cycloparaffins	30.9 Wt. %	33.4 Wt %
Monocycloparaffins	18.8	23.4
Dicycloparaffins	9.3	8.0
Tricycloparaffins	2.8	2.0
Aromatics	27.4 Wt. %	23.9 Wt. %
Alkylbenzenes	8.4	9.7
Indans & tetralins	5.3	4.8
Dinaphthenobenzenes	1.1	1.3
C11 + naphthalenes	7.6	
Biphenyls etc.	2.5	1.3
Fluorenes etc.	1.1	0.7
Tricyclic aromatics	1.4	0.8

DGMK SAMPLES

3 samples: Nos. 22, 23 & 24.

The source of crude and the blend components used for the manufacture of the diesel fuels are detailed in the report. Other characterization parameters are tabulated below.

Parameter	**DGMK Sample No.**		
	22	**23**	**24**
Density (g/ml @ 15°C)	0.8222	0.8433	0.8337
IBP (°C)	161	151	143
FBP (°C)	349	384	347
Viscosity (mm2/sec @40°C)	2.2	3.25	2.11
Carbon (wt%)	85.94	86.4	86.44
Hydrogen (wt%)	13.85	13.35	13.36
Total nitrogen (mg/l)	46	171	125
Total sulfur (wt%)	0.12	0.17	
(mg/kg)			457
Aromatics (vol.%)	23.4	39.6	28.1
Olefins (vol.%)	0	1.0	1.0
Saturates (vol.%)	76.6	59.4	71.9
Flash point (°C)	66	65	58

HOME HEATING OILS

Three samples were prepared by the American Petroleum Institute. They were composed of straight run gas oil (CAS 64741-44-2 predominantly saturates) to which cracked stock (CAS 64741-59-9, predominantly aromatics) was added as shown in the following table.

	API Sample No.		
	78-3/ 83-01	**78-2/ 83-02**	**78-4/ 83-03**
Concentration of cracked stock	10%	30%	50%
API gravity	39.5	38	36.5
Density @15°C)	0.8267	0.834	0.8414
RI (RI units @20°C)	1.462	1.1675	1.4743
Mol wt. (g/mol)	200	197	195
Total S (wt%)	0.08	0.11	0.14

Total N (ppm/wt)	37	67	114
Total Cl_2 (ppm/wt)	6	6	6
Saturates (vol %)	79.2	73.4	67.8
Olefins (vol %)	2.9	4.5	6.1
Aromatics (vol %)	17.9	22.1	26.1
Boiling range (° F)	364-615	366-629	358-640

1.13 REVIEWS

Memo : IARC

Remark : IARC reviewed the available data on distillate fuels and assessed the strength of evidence that the fuels were a carcinogenic risk to man and animals.
The conclusions of the IARC review were:

Evaluation:
There is inadequate evidence for the carcinogenicity in humans of diesel fuels.

There is limited evidence for the carcinogenicity in experimental animals of marine diesel fuel.

There is limited evidence for the carcinogenicity in experimental animals of fuel oil No. 2.

The overall evaluations were:

Marine diesel fuel is possibly carcinogenic to humans (Group 2B)

Distillate (light) diesel fuels are not classifiable as to their carcinogenicity to humans (Group 3)

Distillate (light) fuel oils are not classifiable as to their carcinogenicity to humans (Group 3)

(25)

2.1 MELTING POINT

Method : ASTM D97
GLP : No data
Test substance : Middle distillate fuels

Remark : By definition, melting point is the temperature at which a solid becomes a liquid at normal atmospheric pressure. For complex mixtures like petroleum products, melting point may be characterized by a range of temperatures reflecting the melting points of the individual components. To better describe phase or flow characteristics of petroleum products, the pour point is routinely used. The pour point is the lowest temperature at which movement of the test specimen is observed under prescribed conditions of the test (ASTM 1999). The pour point methodology also measures a "no-flow" point, defined as the temperature of the test specimen at which a wax crystal structure and/or viscosity increase such that movement of the surface of the test specimen is impeded under the conditions of the test (ASTM 1999). Because not all petroleum products contain wax in their composition, the pour point determination encompasses change in physical state (i.e., crystal formation) and/or viscosity property.

Result :

Sample	Pour Point (°C)	Method	Ref.
Automotive Gas Oil	-5	ASTM D97	CONCAWE 1996
Heating Oil	0	ASTM D97	CONCAWE 1996
Distillate Marine Fuel	-6	ASTM D97	CONCAWE 1996
Diesel Fuel Oil (2002)	-50	ASTM D97	Jokuty et al. 2002
Diesel Fuel Oil (Alaska)	-36	ASTM D97	Jokuty et al. 2002
Diesel Fuel Oil (Canada)	-30	ASTM D97	Jokuty et al. 2002
Diesel Fuel Oil (Southern USA)	-14	ASTM D97	Jokuty et al. 2002

Reliability : (2) valid with restrictions
Results of standard method testing was reported in a reliable review dossier and reference database.

(13) (28)

2.2 BOILING POINT

Method : ASTM D86
GLP : No data
Test substance : Middle distillate fuels

Result :

Sample	Boiling Range (°C)	Method	Ref.
Automotive Gas Oil	160-390	ASTM D86	CONCAWE 1996
Heating Oil	160-400	ASTM D86	CONCAWE 1996
Distillate Marine Fuel	170-420	ASTM D86	CONCAWE 1996
Diesel Fuel Oil (Alaska)	141-320	ASTM D86	Jokuty et al. 2002
Diesel Fuel Oil (Canada)	246-388	ASTM D86	Jokuty et al. 2002
Diesel Fuel Oil (USA)	174-352	ASTM D86	Jokuty et al. 2002

Reliability : (2) valid with restrictions
Results of standard method testing was reported in a reliable review dossier and reference database.

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2.4 VAPOUR PRESSURE

Method : Calculated: ASTM D2889
GLP : No data
Test substance : Middle distillate fuels

Remark : Gas oils consist of complex mixtures of various hydrocarbon compounds having diverse structures represented by paraffins, olefins, naphthenes, and aromatics. Molecular weights of these hydrocarbons range between C9 to C30 (CONCAWE 1996; CONCAWE 2001). Although individual hydrocarbon constituents in gas oil exert their own vapor pressures, the vapor pressure of the mixture is the sum of the individual partial pressures of the components. The values given above are considered to be representative of the general category of gas oils.

Result :

Vapor Pressures (kPa)	Method	Ref.
Automotive Gas Oil:		
0.4 (approx.)	ASTM D2889	CONCAWE 1996
Heating Oil:		
0.4 (approx.)	ASTM D2889	CONCAWE 1996
Diesel Fuel Canada:		
2	ASTM D323	Jokuty et al. 2002
Fuel Oil No. 2:		
2	ASTM D323	Jokuty et al. 2002

Reliability : (2) valid with restrictions

(13) (14) (28)

3.5 BIODEGRADATION

Inoculum : Activated sludge
Contact time : 28 day(s)
Method : OECD Guide-line 301 F "Ready Biodegradability: Manometric Respirometry Test"
Year : 2003
GLP : No data
Test substance : Diesel fuel; CAS No. 68334-30-5

Remark : The report states that biodegradation of the test sample achieved 60% by the end of the test, but the classification of readily biodegradable cannot be given to the test sample because it did not achieve the pass level within the 10-day period following 10% biodegradation. However, recent guidance from OECD states that when testing mixtures of structurally related materials such as oils, the 10-day window should not be applied (OECD 2003). Based on the current OECD recommendations, ultra low sulfur diesel is capable of passing a ready biodegradability test.
Although the test substance was not chemically characterized, the report states that ultra low sulfur diesel of the type tested in this study contains 26 - 30% aromatic compounds.

Result : Average biodegradation for duplicate test flasks:

	28 Days	End of 10-d Window
Ultra low sulfur diesel	60	45
Sodium Benzoate (reference)	97	not applicable

Test condition : Activated sludge inoculum for the test was collected from Chester Sewage Works (Welsh Water, Sealand Road, Chester CH1 4LD). The plant treats predominately (approx. 90%) domestic sewage. To reduce background oxygen consumption, the activated sludge was washed then aerated with moist air for approximately 24 hours at the test temperature (20 °C) prior to use. The final inoculum had a concentration of 30 mg dry solids/l.
The diesel fuel was tested in duplicate at a nominal concentration of 30 mg/l, which was equivalent to 102 mg Theoretical Oxygen Demand (ThOD)/l. Test flasks contained 500 ml of inoculated mineral salts medium dosed with 15± 0.5 mg test sample absorbed on a 21 mm Whatman GF/A glass fiber filter. The filter was held on an aluminum foil support during weighing, and both the filter and foil were added to the test vessel. Also included in the experiment were triplicate blank flasks containing inoculated medium and a GF/A filter and aluminum foil and duplicate reference substance flasks containing inoculated medium and 100 mg ThOD/l sodium benzoate (approximately 60 mg/l).
The test was run at 20 °C using a C.E.S. Aerobic Respirometer. The instrument determined the oxygen demand every hour and replenished the oxygen through the electrolysis of copper sulfate. The resolution of the instrument was 0.02 mg oxygen at S.T.P. (standard temperature and pressure).
The extent of biodegradation over 28 days was calculated as the measured biochemical oxygen demand (BOD) expressed as a percentage of the ThOD. The ThOD for diesel fuel was determined previously as 3.4 mg O_2/mg test substance (Battersby 2000). Results were evaluated in light of the pass level for ready biodegradability. The pass level for the OECD 301 F test is = 60% ThOD within the 28-day test period, and it must be attained within 10 days after biodegradation has achieved 10% ThOD.

Reliability : (1) valid without restriction

(9) (12) (30)

Inoculum	:	Activated sludge
Contact time	:	28 day(s)
Method	:	OECD Guide-line 301 F "Ready Biodegradability: Manometric Respirometry Test"
Year	:	1999
GLP	:	No
Test substance	:	Nigerian Diesel fuel CAS No. 68334-30-5
Remark	:	This diesel fuel stream did not satisfy the test criteria for ready degradability of 60% degradability within 28 days. Although this diesel fuel is not considered 'readily' biodegradable, it is inherently biodegradable since significant degradation did occur, based on EPA guidance for using ready and inherent biodegradability tests (http://www.epa.gov/oppt/exposure/docs/halflife.htm.)
Result	:	Average biodegradation for duplicate test flasks at 28 days: diesel fuel = 57.5%; rapeseed oil = 84.4% (satisfied positive control criteria). The report noted that the oxygen consumption of the blank controls was below 60 mg/l as required by the test guidelines.
Test condition	:	Activated sludge inoculum for the test was collected from Medford Municipal Wastewater Treatment Plant in Medford, NJ. The plant treats predominately domestic sewage. To reduce background oxygen consumption, the activated sludge was aerated for approximately 27 hours prior to use. Sufficient supernatant was decanted to provide a 1% (v/v) inoculum for each respirometry vessel. The sewage inoculum had a microbial density of $1E^4$ colony forming units per ml, as measured using a commercial dip-slide method, and was within the guideline criteria of $1E^7$-$1E^8$ CFU/l. Test flasks contained 990 ml of inoculated mineral salts medium, ca. 25 mg of test substance and 10 ml of sludge supernatant (inoculum). Also included in the experiment were duplicate blank flasks containing inoculated medium and duplicate reference substance flasks containing inoculated medium and 25 mg of low erucic acid rapeseed oil (LEAR). The test was run at 22 °C (±1 °C) using a C.E.S. Aerobic Respirometer for 28 days. The instrument determined the oxygen demand every hour and replenished the oxygen through the electrolysis of copper sulfate. The extent of biodegradation over 28 days was calculated as the measured biochemical oxygen demand (BOD) expressed as a percentage of the ThOD (theoretical oxygen demand).
Reliability	:	(1) valid without restriction

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4.1 ACUTE/PROLONGED TOXICITY TO FISH

Type : Fish Acute Toxicity Test w/ 24 Hr Renewal
Species : Cyprinodon variegatus (Fish, estuary, marine)
Exposure period : 96 hour(s)
Unit : mg/l
LL50 : 57 measured/nominal
Analytical monitoring : Yes
Method : EPA/600/4-90/027 Methods for Measuring Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms.
Year : 1998
GLP : No
Test substance : CAS No. 68476-30-2; No.2 fuel oil

Method : Statistical Method: Trimmed Spearman Karber Method.
Remark : Data have been developed which allow the quantification of complex hydrocarbons in water on a molar basis using SPME. (Parkerton, T.F., et al., Biomimetic, Extraction as a Cost-Effective Analytical Tool for Determining the Aquatic Toxicity Hazard of Complex Petroleum Products SETAC Europe, 2001). The measured molar concentrations reflect those portions that are potentially bioaccumulative in aquatic and terrestrial species. SPME is a surrogate for organism lipid and since it is measuring dissolved, unbound hydrocarbon, the BPH concentration on the fiber is a direct measure of potential toxicity. Data developed at EMBSI comparing SPME molar concentrations of fuel oil in WAFs versus fuel oil toxicity assessed with a wide variety of aquatic species indicates that SPME quantification of bioavailable petroleum hydrocarbons (BPH) in fuel oil correlates well with observed aquatic toxicity. The total molar sum of components that partition to the fiber from the aqueous phase of a complex mixture in a lethal loading test "mimics" the total body residue in an aquatic test organism. Acute toxicity is predicted once a critical threshold on the SPME fiber (Cfiber,critical, also BPH critical) is exceeded. Thus, the Cfiber, critical provides a simple analytical measure that is comparable to the narcosis-based critical body residue (CBR) for a given test organism/endpoint.

Result :

Nominal Conc. (mg/l)	BPH (nmoles/mgC)	% Mortality 24hr	48 hr	72 hr	96hr
Control	-----	0	0	0	0
9.9	88	0	0	0	0
32	186	0	0	0	0
60.0	192	0	0	20	60
124	257	0	80	100	100

Based on nominal loading rates:
96-hr LL_{50}= 57 mg/l
95% confidence interval 48-68 mg/l
CBR= Cfiber,critical = 73 µmol/mlPDMS
BPH critical = 202 nmol/mg C

Test condition : Nominal loading rates of 0, 9.9, 32, 60 and 124 mg/l were used to prepare test solutions. Test solutions were prepared as water accommodated fractions (WAF). Control and dilution water were natural seawater (20 ppt salinity) obtained from Manasquan Inlet, Manasquan, New Jersey. Test substance was mixed for each individual treatment in dilution water for 24 hours in stoppered containers with less than 10% headspace volume. The mixtures were allowed to settle for 1-2 hours prior to drawing off the aqueous solutions for testing. BPH analysis was performed using SPME

(solid phase microextraction) fibers which had been equilibrated overnight in WAF solutions and quantified using gas chromatography equipped with a flame ionization detector. Fish were 11 days old at initiation of testing, and were obtained from Aquatic Systems, Inc., Fort Collins, CO. Test vessels were 500 ml glass I-Chem jars with Teflon lined caps. Three replicates per treatment and 4 organisms per replicate were tested for each treatment and the control. Exposure containers were filled with no headspace and tightly sealed to prevent volatilization. Test solution renewal was performed daily by removing 80% of the test solution and replacing it with fresh WAF solution prepared at least 24 hrs prior to use. Water temperature was 24.8 °C. Test photoperiod was 16 hrs. light and 8 hr dark, light intensity approx 69 foot-candles during full daylight periods. Dissolved oxygen measurements ranged from 5.0 to 7.7 ppm, pH values between 7.6 and 8.3. Duplicate samples were taken of the control on Day 0 and each WAF (from the mixing vessel) prepared on a daily basis. Samples were used to determine bioavailable petroleum hydrocarbons (BPH).
Measurement of dissolved, bioavailable hydrocarbon was performed using solid phase microextraction (SPME) fiber analysis by GC/FID. BPH samples were mixed (minimal headspace) in vessels containing the SPME fibers overnight.
Analyses were performed using 100 um PDMS fibers obtained from Supelco. 125 ml glass flasks fitted with ground glass stoppers and equipped with stir bars were filled with WAF samples. SPME fibers were inserted through a small hole drilled in the center of the glass stopper. The total moles of hydrocarbons that partition to the fibers were quantified using a gas chromatograph equipped with a flame ionization detector (GC-FID). FID detector response was translated into molar hydrocarbon concentration (BPH as nmoles/mg C) using the molar response based on a number of aromatic standards. BPH values are reported as nanomoles of quantified hydrocarbon per mg of carbon based on carbon content of polydimethylsiloxane (PDMS) fiber coating. Critical SPME fiber concentration (Cfiber, critical), comparable to critical total body residue (CBR, on a lipid basis) is calculated from BPH results and is reported in units of µmoles/ml of PDMS. Quantitation was based on 2, 3 dimethyl napthalene since a wide variety of hydrocarbons exhibited a relative molar response of within a factor of two when normalized to this compound. Calculation of CBR as Cfiber, critical is based on Verbuggen et al.

Reliability : (2) Reliable with restrictions. The data reported in this report were obtained as part of a research program to correlate toxicity with Bioavailable Petroleum hydrocarbons (BPH). Thus although the work was conducted according to relevant test guidelines and standard operating procedures, the research was not done in strict accordance with GLPs. All data reported as final were audited by EBSI Quality Assurance unit.

(21) (39)

Type	:	Fish Acute Toxicity Test w/ 24 Hr Renewal
Species	:	Menidia beryllina (Fish, estuary, marine)
Exposure period	:	96 hour(s)
Unit	:	mg/l
LL50	:	3.2 measured/nominal
Analytical monitoring	:	Yes
Method	:	EPA/600/4-90/027 Methods for Measuring Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms.
Year	:	1995
GLP	:	No

Test substance : other TS: CAS No. 68476-30-2; No.2 fuel oil

Method : Statistical Method: Trimmed Spearman Karber Method.

Remark : Data have been developed which allow the quantification of complex hydrocarbons in water on a molar basis using SPME. (Parkerton, T.F., et al., Biomimetic Extraction as a Cost-Effective Analytical Tool for Determining the Aquatic Toxicity Hazard of Complex Petroleum ProductsSETAC Europe, 2001). The measured molar concentrations reflect those portions that are potentially bioaccumulative in aquatic and terrestrial species. SPME is a surrogate for organism lipid and since it is measuring dissolved, unbound hydrocarbon, the BPH concentration on the fiber is a direct measure of potential toxicity. Data developed at EMBSI comparing SPME molar concentrations of fuel oil in WAFs versus fuel oil toxicity assessed with a wide variety of aquatic species indicates that SPME quantification of bioavailable petroleum hydrocarbons (BPH) in fuel oil correlates well with observed aquatic toxicity. The total molar sum of components that partition to the fiber from the aqueous phase of a complex mixture in a lethal loading test "mimics" the total body residue in an aquatic test organism. Acute toxicity is predicted once a critical threshold on the SPME fiber (Cfiber,critical, also BPH critical) is exceeded. Thus, the Cfiber, critical provides a simple analytical measure that is comparable to the narcosis-based critical body residue (CBR) for a given test organism/endpoint.

Result : **Nominal**

Conc.	**BPH**		**% Mortality**		
(mg/l)	**(nmoles/mgC)**	**24hr**	**48 hr**	**72 hr**	**96hr**
Control	-----	0	0	0	0
1.25	37.2	0	0	0	0
2.9	50.3	0	7	13	13
5.2	78.1	0	67	100	100
10.0	114.4	13	100	100	100

Based on nominal loading rates:
96-hr LL_{50}= 3.2 mg/l, 95% confidence interval 2.9-3.6 mg/l
CBR= Cfiber,critical = 26 µmol/mlPDMS
BPH critical = 72 nmol/mg C

Test condition : Nominal loading rates of 0, 1.25, 2.9, 5.2 and 10.0 mg/l were used to prepare test solutions. Test solutions were prepared as water accommodated fractions (WAF). Control and dilution water were natural seawater (20 ppt salinity) obtained from Manasquan Inlet, Manasquan, New Jersey. Test substance was mixed for each individual treatment in dilution water for 24 hours in stoppered containers with less than 10% headspace volume. The mixtures were allowed to settle for 1-2 hours prior to drawing off the aqueous solutions for testing. BPH analysis was performed using SPME (solid phase microextraction) fibers which had been equilibrated overnight in WAF solutions and quantified using gas chromatography equipped with a flame ionization detector. Fish were 11 days old at initiation of testing, and were obtained from Aquatic Systems, Inc., Fort Collins, CO. Test vessels were 500 ml glass I-Chem jars with teflon lined caps. Three replicates per treatment and 5 organisms per replicate were tested for each treatment and the control.
Exposure containers were filled with no headspace and tightly sealed to prevent volatilization. Test solution renewal was performed daily by removing 80% of the test solution and replacing it with fresh WAF solution prepared at least 24 hrs prior to use. Water temperature was 24.8 °C. Test photoperiod was 16 hrs. light and 8 hr dark, light intensity approx 69 foot-candles during full daylight periods. Dissolved oxygen measurements ranged from 5.3 to 7.7 ppm, pH values between 7.7 and 8.4.Duplicate

samples were taken of the control on Day 0 and each WAF (from the mixing vessel) prepared on a daily basis. Samples were used to determine bioavailable petroleum hydrocarbons (BPH).
Measurement of dissolved, bioavailable hydrocarbon was performed using solid phase microextraction (SPME) fiber analysis by GC/FID. BPH samples were mixed (minimal headspace) in vessels containing the SPME fibers overnight. Analyses were performed using 100 um PDMS fibers obtained from Supelco. 125 ml glass flasks fitted with ground glass stoppers and equipped with stir bars were filled with WAF samples. SPME fibers were inserted through a small hole drilled in the center of the glass stopper. The total moles of hydrocarbons that partition to the fibers were quantified using a gas chromatograph equipped with a flame ionization detector (GC-FID). FID detector response was translated into molar hydrocarbon concentration (BPH as nmoles/mg C) using the molar response based on a number of aromatic standards.
BPH values are reported as nanomoles of quantified hydrocarbon per mg of carbon based on carbon content of polydimethylsiloxane (PDMS) fiber coating. Critical SPME fiber concentration (Cfiber, critical), comparable to critical total body residue (CBR, on a lipid basis) is calculated from BPH results and is reported in units of µmoles/ml of PDMS. Quantitation was based on 2, 3 dimethyl napthalene since a wide variety of hydrocarbons exhibited a relative molar response of within a factor of two when normalized to this compound. Calculation of CBR as Cfiber, critical is based on Verbuggen et al.

Reliability : (2) Reliable with restrictions.
The data reported in this report were obtained as part of a research program to correlate toxicity with Bioavailable Petroleum hydrocarbons (BPH). Thus although the work was conducted according to relevant test guidelines and standard operating procedures, the research was not done in strict accordance with GLPs. All data reported as final were audited by EBSI Quality Assurance unit.

(19) (39)

Type : Fish Acute Toxicity Test w/ 24 Hr Renewal
Species : Oncorhynchus mykiss (Fish, fresh water)
Exposure period : 96 hour(s)
Unit : mg/l
LL50 : 6.6 measured/nominal
Analytical monitoring : Yes
Method : OECD Guide-line 203 "Fish, Acute Toxicity Test"
Year : 1995
GLP : No
Test substance : CAS No. 68476-30-2; No.2 fuel oil

Method : Statistical Method: Trimmed Spearman Karber Method, 1977.
Remark : Data have been developed which allow the quantification of complex hydrocarbons in water on a molar basis using SPME. (Parkerton, T.F., et al., Biomimetic Extraction as a Cost-Effective Analytical Tool for Determining the Aquatic Toxicity Hazard of Complex Petroleum Products, SETAC Europe, 2001). The measured molar concentrations reflect those portions that are potentially bioaccumulative in aquatic and terrestrial species. SPME is a surrogate for organism lipid and since it is measuring dissolved, unbound hydrocarbon, the BPH concentration on the fiber is a direct measure of potential toxicity. Data developed at EMBSI comparing SPME molar concentrations of fuel oil in WAFs versus fuel oil toxicity assessed with a wide variety of aquatic species indicates that SPME

quantification of bioavailable petroleum hydrocarbons (BPH) in fuel oil correlates well with observed aquatic toxicity. The total molar sum of components that partition to the fiber from the aqueous phase of a complex mixture in a lethal loading test "mimics" the total body residue in an aquatic test organism. Acute toxicity is predicted once a critical threshold on the SPME fiber (Cfiber,critical; also BPH critical) is exceeded. Thus, the Cfiber,critical provides a simple analytical measure that is comparable to the narcosis-based critical body residue (CBR) for a given test organism/endpoint.

Result :

Nominal Conc. (mg/l)	BPH (nmoles/mgC)	% Mortality 24hr	48 hr	72 hr	96hr
Control	0.2	0	0	0	0
2.3	79.7	0	0	0	0
4.9	102.7	0	0	7	7
9.8	183.4	7	7	50	100
23.2	261.9	43	100	100	100
49.8	314.3	100	100	100	100

Based on nominal loading rates: 96hr LL_{50}= 6.6 mg/l
95% confidence interval, 6.0-7.3 mg/l,
CBR= Cfiber,critical = 56 µmol/mlPDMS
BPH critical = 155 nmol/mg C

Test condition : Nominal loading rates of 0, 2.3, 4.9, 9.8, 23.2 and 49.8 mg/l were used to prepare test solutions. Test solutions were prepared as water accommodated fractions (WAF). The control and dilution water was a laboratory blend water prepared from carbon filtered well water and ion exchange softened, reverse osmosis dialyzed well water aged for >24 hrs. Test substance was mixed for each individual treatment in dilution water for 24 hours in stoppered containers with less than 10% headspace volume. The mixtures were allowed to settle for 1-2 hours prior to drawing off the aqueous solutions for testing. BPH analysis was performed using SPME (solid phase microextraction) fibers which had been equilibrated overnight in WAF solutions and quantified using gas chromatography equipped with a flame ionization detector. Fish were approximately three weeks old at initiation of testing, and were obtained from Thomas Fish company, Anderson, CA. Loading of fish body mass to treatment was 0.193g fish per liter of aqueous solution. Test vessels were 4L glass aspirator bottles with Teflon covered neoprene stoppers. Two replicates per treatment and 7 organisms per replicate were tested for each treatment and the control. Exposure containers were filled with no headspace and tightly sealed to prevent volatilization. Test solution renewal was performed daily by removing 80% of the test solution and replacing it with fresh WAF solution prepared at least 24 hrs prior to use. Water temperature was 14.1 °C (0.7sd). Test photoperiod was 16 hrs. light and 8 hr dark, light intensity approx 69 foot-candles during full daylight periods. Dissolved oxygen measurements ranged from 6.9 to 8.2 ppm, pH values between 6.6 and 7.2. Duplicate samples were taken of the control on Day 0 and each WAF (from the mixing vessel) prepared on a daily basis. Samples were used to determine bioavailable petroleum hydrocarbons (BPH). Measurement of dissolved, bioavailable hydrocarbon was performed using solid phase microextraction (SPME) fiber analysis by GC/FID. BPH samples were mixed (minimal headspace) in vessels containing the SPME fibers overnight.
Analyses were performed using 100 um PDMS fibers obtained from Supleco. 125 ml glass flasks fitted with ground glass stoppers and equipped with stir bars were filled with WAF samples. SPME fibers were inserted through a small hole drilled in the center of the glass stopper. The total moles of hydrocarbons that partition to the fibers were quantified using

a gas chromatograph equipped with a flame ionization detector (GC-FID). FID detector response was translated into molar hydrocarbon concentration (BPH as nmoles/mg C) using the molar response based on a number of aromatic standards.
BPH values are reported as nanomoles of quantified hydrocarbon per mg of carbon based on carbon content of polydimethylsiloxane (PDMS) fiber coating. Critical SPME fiber concentration (Cfiber, critical), comparable to critical total body residue (CBR, on a lipid basis) is calculated from BPH results and is reported in units of µmoles/ml of PDMS. Quantitation was based on 2, 3 dimethyl napthalene since a wide variety of hydrocarbons exhibited a relative molar response of within a factor of two when normalized to this compound. Calculation of CBR as Cfiber, critical is based on Verbruggen et al.

Reliability : (2) Reliable with restrictions.
The data reported in this report were obtained as part of a research program to correlate toxicity with Bioavailable Petroleum Hydrocarbons (BPH). Thus although the work was conducted according to relevant test guidelines and standard operating procedures, the research was not done in strict accordance with GLPs. All data reported as final were audited by EBSI Quality Assurance unit.

(20) (39)

Type : Fish Acute Toxicity Test w/ 24 Hr Renewal
Species : Pimephales promelas (Fish, fresh water)
Exposure period : 96 hour(s)
Unit : mg/l
LL50 : 57 measured/nominal
Analytical monitoring : Yes
Method : EPA/600/4-90/027 Methods for Measuring Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms.
Year : 1998
GLP : No
Test substance : CAS No. 68476-30-2; No.2 fuel oil

Method : Statistical Method: Trimmed Spearman Karber Method.
Remark : Data have been developed which allow the quantification of complex hydrocarbons in water on a molar basis using SPME. (Parkerton, T.F., et al., Biomimetic Extraction as a Cost-Effective Analytical Tool for Determining the Aquatic Toxicity Hazard of Complex Petroleum Products, SETAC Europe, 2001). The measured molar concentrations reflect those portions that are potentially bioaccumulative in aquatic and terrestrial species. SPME is a surrogate for organism lipid and since it is measuring dissolved, unbound hydrocarbon, the BPH concentration on the fiber is a direct measure of potential toxicity. Data developed at EMBSI comparing SPME molar concentrations of fuel oil in WAFs versus fuel oil toxicity assessed with a wide variety of aquatic species indicates that SPME quantification of bioavailable petroleum hydrocarbons (BPH) in fuel oil correlates well with observed aquatic toxicity. The total molar sum of components that partition to the fiber from the aqueous phase of a complex mixture in a lethal loading test "mimics" the total body residue in an aquatic test organism. Acute toxicity is predicted once a critical threshold on the SPME fiber (Cfiber,critical, also BPH critical) is exceeded. Thus, the Cfiber, critical provides a simple analytical measure that is comparable to the narcosis-based critical body residue (CBR) for a given test organism/endpoint.

Result : **Nominal**

Conc. (mg/l)	BPH (nmoles/mgC)	% Mortality 24hr	48 hr	72 hr	96hr
Control	----	0	0	0	0
41	353	0	0	0	0
78	426.5	33	33	33	100
169	473.1	83	92	100	100
326	573.1	100	100	100	100
612	594.2	58	100	100	100

Based on nominal loading rates: 96-hr LL_{50}= 57mg/l
95% confidence interval 41-78 mg/l
CBR= Cfiber,critical = 140 µmol/mlPDMS,
BPH critical = 388 nmol/mg C

Test condition : Nominal loading rates of 0, 41, 78, 169, 326 and 612 mg/l were used to prepare test solutions. Test solutions were prepared as water accommodated fractions (WAF). The control and dilution water was a laboratory blend water prepared from carbon filtered well water and ion exchange softened, reverse osmosis dialyzed well water aged for >24 hrs. Test substance was mixed for each individual treatment in dilution water for 24 hours in stoppered containers with less than 10% headspace volume. The mixtures were allowed to settle for 1-2 hours prior to drawing off the aqueous solutions for testing. BPH analysis was performed using SPME (solid phase microextraction) fibers which had been equilibrated overnight in WAF solutions and quantified using gas chromatography equipped with a flame ionization detector. Fish were approximately between 6-8 weeks old at initiation of testing, and were obtained from Aquatic Systems, Inc., Fort Collins, CO. Test vessels were 500 ml glass I-Chem jars with teflon lined caps. Three replicates per treatment and 4 organisms per replicate were tested for each treatment and the control. Exposure containers were filled with no headspace and tightly sealed to prevent volatilization. Test solution renewal was performed daily by removing 80% of the test solution and replacing it with fresh WAF solution prepared at least 24 hrs prior to use. Water temperature was 24.9 °C (0.1°C sd). Test photoperiod was 16 hrs. light and 8 hr dark, light intensity approx 69 foot-candles during full daylight periods. Dissolved oxygen measurements >60% saturation, pH values between 6.4 and 8.8. Duplicate samples were taken of the control on Day 0 and each WAF (from the mixing vessel) prepared on a daily basis.

Samples were used to determine bioavailable petroleum hydrocarbons (BPH). Measurement of dissolved, bioavailable hydrocarbon was performed using solid phase microextraction (SPME) fiber analysis by GC/FID. BPH samples were mixed (minimal headspace) in vessels containing the SPME fibers overnight. Analyses were performed using 100 um PDMS fibers obtained from Supelco. 125 ml glass flasks fitted with ground glass stoppers and equipped with stir bars were filled with WAF samples. SPME fibers were inserted through a small hole drilled in the center of the glass stopper. The total moles of hydrocarbons that partition to the fibers were quantified using a gas chromatograph equipped with a flame ionization detector (GC-FID). FID detector response was translated into molar hydrocarbon concentration (BPH as nmoles/mg C) using the molar response based on a number of aromatic standards. BPH values are reported as nanomoles of quantified hydrocarbon per mg of carbon based on carbon content of polydimethylsiloxane (PDMS) fiber coating. Critical SPME fiber concentration (Cfiber, critical), comparable to critical total body residue (CBR, on a lipid basis) is calculated from BPH results and is reported in units of µmoles/ml of PDMS. Quantitation was based on

2, 3 dimethyl napthalene since a wide variety of hydrocarbons exhibited a relative molar response of within a factor of two when normalized to this compound.
Calculation of CBR as Cfiber, critical is based on Verbuggen et al.

Reliability : (2) Reliable with restrictions.
The data reported in this report were obtained as part of a research program to correlate toxicity with Bioavailable Petroleum Hydrocarbons (BPH). Thus although the work was conducted according to relevant test guidelines and standard operating procedures, the research was not done in strict accordance with GLPs. All data reported as final were audited by EBSI Quality Assurance unit.

(22) (39)

Type : Semistatic
Species : Oncorhynchus mykiss (Fish, fresh water)
Exposure period : 96 hour(s)
Unit : mg/l
LL50 : 21 measured/nominal
Analytical monitoring : Yes
Method : OECD Guide-line 203 "Fish, Acute Toxicity Test"
Year : 1995
GLP : Yes
Test substance : CAS No. 68334-30-5; Gas oil

Method : Statistical Method: Moving average angle method
Result : 96-hr LL_{50} = 21 mg/l, 95% confidence interval of 12 – 40 mg/l based on nominal loading rates.

Mortality at 96 hrs was 1, 0, 0, 6, and 7 in the 0, 3, 10, 30, and 100 mg/l treatments. Only four concentrations were tested which is less than a minimum of five concentrations stated in the guidelines. Water hardness was higher than targeted range of 50 - 250 mg/l as $CaCO_3$. Hardness range of 264 - 288 mg/l as $CaCO_3$ is normal for this laboratory and does not adversely affect the health of the fish. Analytical method used was gas chromatography-mass spectrometry. Mean reduction in the concentration of dissolved hydrocarbons during the test was 26% (range 9 - 42%).

Test condition : Individual treatment concentrations were prepared as water accommodated fractions (WAF). Nominal loading rates in the definitive test were 0, 3, 10, 30, 100 mg/l. Control and dilution water was laboratory mains tap water obtained from bore holes, and passed through particle and activated carbon filters (alkalinity 257 mg/l as $CaCO_3$, hardness 284 mg/l as $CaCO_3$, conductivity 496 µS/cm, pH 7.5). Test substance was mixed in dilution water for ~72 hrs in sealed vessels with minimal headspace. Mixing time was determined in an equilibration study in which the test substance concentration in the aqueous phase of the WAFs was monitored by GC-MS. Mixtures were allowed to settle 1.5 to 2 hrs prior to drawing off the aqueous phase for testing. Test vessels were sealed 11-liter glass aspirators with 7 fish per vessel. Test fish had a mean length of 5.5 cm and a mean weight of 1.2 g. Fingerlings were obtained from Exmoor Trout Farm, North Molton, Devon, U.K. One replicate per treatment and control were used. Test solutions were renewed daily with surviving fish transferred to the freshly prepared WAFs. Test temperature was 15.4 - 16.2 °C. Photoperiod was 16 hrs light and 8 hrs dark. Dissolved oxygen was >60% saturation (8.7 to 9.5 mg/l). pH was 7.0 - 7.8. To monitor the concentration of soluble components in the test solutions, samples were

collected at the beginning and end of each 24-hr period for each of the batches of WAFs prepared during the 96-hr test.

Reliability : (1) valid without restriction

(37)

Type : Semistatic
Species : Oncorhynchus mykiss (Fish, fresh water)
Exposure period : 96 hour(s)
Unit : mg/l
Analytical monitoring : Yes
Method : OECD Guide-line 203 "Fish, Acute Toxicity Test"
Year : 1995
GLP : Yes
Test substance : CAS No. 68334-30-5; Gas oil

Method : Statistical Method: Moving average angle method
Result : 96-hr LL_{50} = 65 mg/l, 95% confidence interval of 21 – 290 mg/l based on nominal loading rates.

Mortality at 96 hrs was 0, 0, 0, 5, and 7 in the 0, 1, 10, 100, and 1000 mg/l treatments. Only four concentrations were tested which is less than a minimum of five concentrations stated in the guidelines. Dilution factor of 10 is greater than recommended. Water hardness was higher than targeted range of 50 - 250 mg/l as $CaCO_3$. Hardness range of 270 – 288 mg/l as $CaCO_3$ is normal for this laboratory and does not adversely affect the health of the fish. Analytical method used was gas chromatography-mass spectrometry. Mean reduction in the concentration of dissolved hydrocarbons during the test was 27% (range 10 - 50%).

Test condition : Individual treatment concentrations were prepared as water accommodated fractions (WAF). Nominal loading rates in the definitive test were 0, 1, 10, 100, 1000 mg/l. Control and dilution water was laboratory mains tap water obtained from bore holes, and passed through particle and activated carbon filters (alkalinity 257 mg/l as $CaCO_3$, hardness 284 mg/l as $CaCO_3$, conductivity 496 µmS/cm, pH 7.5). Test substance was mixed in dilution water for ~70 hrs. Mixing time was determined in an equilibration study in which the test substance concentration in the aqueous phase of the WAFs was monitored by GC-MS. Mixtures were allowed to settle 1.5 to 2 hrs prior to drawing off the aqueous phase for testing. Test vessels were sealed 11-liter glass aspirators with 7 fish per vessel. Test fish had a mean length of 5.6 cm and a mean weight of 1.2 g. Fingerlings were obtained from Exmoor Trout Farm, North Molton, Devon, U.K. One replicate per treatment and control were used. Test solutions were renewed daily with surviving fish transferred to the freshly prepared WAFs. Test temperature was 15.0 - 16.0 °C. Photoperiod was 16 hrs light and 8 hrs dark. Dissolved oxygen was >60% saturation (8.8 to 9.2 mg/l). pH was 7.2 - 7.5. To monitor the concentration of soluble components in the test solutions, samples were collected at the beginning and end of each 24-hr period for each of the batches of WAFs prepared during the 96-hr test.

Reliability : (2) Reliable with restrictions.
Dilution factor of 10 used in the definitive test concentrations was several fold higher than guidelines recommendation and resulted in a wide 95% confidence interval for the 96-hr LL_{50}.

(36)

4.2 ACUTE TOXICITY TO AQUATIC INVERTEBRATES

Type : Static
Species : Daphnia magna (Crustacea)
Exposure period : 48 hour(s)
Unit : mg/l
EL50 : 7.81 measured/nominal
Analytical monitoring : Yes
Method : OECD Guide-line 202
Year : 2000
GLP : Yes
Test substance : CAS 68476-30-2, No. 2 Fuel oil (gas oil, unspecified)

Method : Statistical Method: Probit analysis, Finney. D.J. Stat. Method in Biological Assay. 2nd ed. London, 1984.

Result : Number of immobilized daphnids after 48 hours were:

Treatment conc. (mg/l)	No. immobilized Daphnids
0 (control)	1
0.625	0
1.25	0
2.5	2
5.0	9
10.0	10

48-hr EL_{50} = 7.81 mg/l based upon nominal loading rate, 95% C.I. range 48-hr EL_{50} = 5.00-10.02 mg/l loading

Source : Study sponsor-DGMK, Deutsche Wissenschaftliche Gesellschaft fur Erdol, Erdgas und Kohle (German Research Organization for Oil, Gas and Coal). Distributed by CONCAWE

Test condition : Test solutions were prepared as water accommodated fractions (WAFs). Control and dilution water were purified, sterilized drinking water. Purification includes filtration with charcoal, aeration and passage through a lime stone column. This final step may be a factor causing high, but apparently non-toxic pH values for the purified water (>8.5 to 9.88). Equilibration studies were conducted for a diesel and fuel oil at 100 and 1000 mg/l loading. WAFS were prepared in duplicate by mixing the appropriate mass of substance in 2 liter of water for 70 hr in sealed brown glass screw top bottles fitted w/ a port near the bottle bottom for drawing off WAF samples for GC analysis. An equilibration time of 21 hr was deemed sufficient to achieve maximum aqueous phase saturation of the gas oils. Range finding toxicity studies were conducted for all gas oils at 10 and 100 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Definitive toxicity studies were conducted at 0.625, 1.25, 2.5, 5.0 & 10.0 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Test vessels were sealed 200 ml conical flasks with10 daphnids per flask and were completely filled with test solution. Dissolved oxygen was >60% saturation during the study. The pH was 9.11 at test start and 8.97 at the end (high value may be a concern). Temperature was 20.1-20.5 °C. Daphnia magna, STRAUS clone 5 organisms were supplied by testing laboratory; age < 24 hours old; second generation collected from cultures aged at least 3 weeks old.

Analytical monitoring confirmed consistency of solubilized components of gas oil in aqueous solution, and was also used during WAF preparation to evaluate equilibration period required for maximum solubility of

components. GC/FID analysis of cyclohexane extracts of WAF was based on ISO/DIS 9377-4:1999 method. The individual peak areas of four significant components detected in the WAF extracts were compared to the peak area of the internal standard, n-tetratriacontane. The ratios of each of the four component peak areas to internal standard peak area measured during the equilibration test remained constant after 21 hours of mixing, indicating this was sufficient time to reach maximum solubility for those hydrocarbons with affinity to partition to the aqueous phase. This normalization of peak area was also used to confirm stability of the WAF during the acute range finder and definitive toxicity tests.

Test substance : HEL-blend: middle range distillate; 44% Coker (+hydrotreating), 15% FCC (+hydrotreating), 41% straight run; sample ID A-2133

Reliability : (2) Reliable with restriction due to high pH value of purified water, ie, values >8.5 for all treatments up to 9.88. However mortality was <10% for control treatments tested concurrently with each of the eight WAFS (5% was observed for one control treatment, no mortality for any other control tests). The high pH may not be of concern, given that the organisms are cultured using this purified water.

(24)

Type : Static
Species : Daphnia magna (Crustacea)
Exposure period : 48 hour(s)
Unit : mg/l
EL50 : 12.99 measured/nominal
Analytical monitoring : Yes
Method : OECD Guide-line 202
Year : 2000
GLP : Yes
Test substance : CAS No. 68476-34-6; diesel fuel (Gas oil, unspecified

Method : Statistical method: Probit analysis, Finney. D.J. Stat. Method in Biological Assay. 2nd ed. London, 1984.

Result : Number of immobilized daphnids after 48 hours were 0, 5, 12, 14, 15, and 17 in the control, 6.25, 12.5, 25, 50 & 100 mg/l treatments, respectively. 48-hr EL_{50} = 12.99 mg/l based upon nominal loading rate,95% C.I. range 48-hr EL_{50} = 7.53-22.41 mg/l loading

Source : Study sponsor-DGMK, Deutsche Wissenschaftliche Gesellschaft fur Erdol, Erdgas und Kohle (German Research Organization for Oil, Gas and Coal). Distributed by CONCAWE.

Test condition : Test solutions were prepared as water accommodated fractions (WAFs). Control and dilution water were purified, sterilized drinking water. Purification includes filtration with charcoal, aeration and passage through a lime stone column. This final step may be a factor causing high, but apparently non-toxic pH values for the purified water (>8.5 to 9.88). Equilibration studies were conducted for a diesel and fuel oil at 100 and 1000 mg/l loading. WAFS were prepared in duplicate by mixing the appropriate mass of substance in 2liter of water for 70 hr in sealed brown glass screw top bottles fitted w/ a port near the bottle bottom for drawing off WAF samples for GC analysis. An equilibration time of 21 hr was deemed sufficient to achieve maximum aqueous phase saturation of the gas oils. Range finding toxicity studies were conducted for all gas oils at 10 and 100 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Definitive toxicity studies were conducted at 6.25, 12.5, 25, 50 & 100 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Test vessels were sealed 200 ml conical flasks with10

daphnids per flask and were completely filled with test solution. Dissolved oxygen was >60% saturation during the study. The pH of the test system solutions ranged from 9.59 to 9.74 (high value may be concern). Temperature was 20.0-20.6 °C. Daphnia magna, STRAUS clone 5 organisms were supplied by testing laboratory; age < 24 hours old; second generation collected from cultures aged at least 3 weeks old.

Analytical monitoring confirmed consistency of solubilized components of gas oil in aqueous solution, and was also used during WAF preparation to evaluate equilibration period required for maximum solubility of components. GC/FID analysis of cyclohexane extracts of WAF was based on ISO/DIS 9377-4:1999 method. The individual peak areas of four significant components detected in the WAF extracts were compared to the peak area of the internal standard, n-tetratriacontane. The ratios of each of the four component peak areas to internal standard peak area measured during the equilibration test remained constant after 21 hours of mixing, indicating this was sufficient time to reach maximum solubility for those hydrocarbons with affinity to partition to the aqueous phase. This normalization of peak area was also used to confirm stability of the WAF during the acute range finder and definitive toxicity tests.

Test substance : 10% Kero, 53% hydrotreated LGO, 37% hydrotreated HGO; sample ID A-2145

Reliability : (2) Reliable with restriction due to high pH value of purified water, i.e., values >8.5 for all treatments up to 9.88. However mortality was <10% for control treatments tested concurrently with each of the eight WAFS (5% was observed for one control treatment, no mortality for any other control tests). The high pH may not be of concern, given that the organisms are cultured using this purified water.

(24)

Type : Static
Species : Daphnia magna (Crustacea)
Exposure period : 48 hour(s)
Unit : mg/l
EL50 : 5.3 measured/nominal
Analytical monitoring : Yes
Method : OECD Guide-line 202
Year : 2000
GLP : Yes
Test substance : CAS No. 68476-30-2; No. 2 fuel oil (Gas oil, unspecified)

Method : Statistical Method: Probit analysis, Finney. D.J. Stat. Method in Biological Assay. 2nd ed. London, 1984.

Result : Number of immobilized daphnids after 48 hours were 0, 0, 0, 7, 8, and 14 in the control, 0.625, 1.25, 2.5, 5.0 & 10.0 mg/l treatments, respectively.48-hr EL_{50} = 5.30 mg/l based upon nominal loading rate,95% C.I. range 48-hr EL_{50} = 3.22-8.71 mg/l loading

Source : Study sponsor-DGMK, Deutsche Wissenschaftliche Gesellschaft fur Erdol, Erdgas und Kohle (German Research Organization for Oil, Gas and Coal). Distributed by CONCAWE.

Test condition : Test solutions were prepared as water accommodated fractions (WAFs). Control and dilution water were purified, sterilized drinking water. Purification includes filtration with charcoal, aeration and passage through a lime stone column. This final step may be a factor causing high, but apparently non-toxic pH values for the purified water (>8.5 to 9.88). Equilibration studies were conducted for a diesel and fuel oil at 100 and

1000 mg/l loading. WAFS were prepared in duplicate by mixing the appropriate mass of substance in 2 liters of water for 70 hr in sealed brown glass screw top bottles fitted w/ a port near the bottle bottom for drawing off WAF samples for GC analysis. An equilibration time of 21 hr was deemed sufficient to achieve maximum aqueous phase saturation of the gas oils. Range finding toxicity studies were conducted for all gas oils at 10 and 100 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Definitive toxicity studies were conducted at 0.625, 1.25, 2.5, 5.0 & 10.0 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Test vessels were sealed 200 ml conical flasks with 10 daphnids per flask and were completely filled with test solution. Dissolved oxygen was >60% saturation during the study. The pH was 9.20 through 9.38 (high value may be concern). Temperature was 20.1-20.5 °C. Daphnia magna, STRAUS clone 5 organisms were supplied by testing laboratory; age < 24 hours old; second generation collected from cultures aged at least 3 weeks old.

Analytical monitoring confirmed consistency of solubilized components of gas oil in aqueous solution, and was also used during WAF preparation to evaluate equilibration period required for maximum solubility of components. GC/FID analysis of cyclohexane extracts of WAF was based on ISO/DIS 9377-4:1999 method. The individual peak areas of four significant components detected in the WAF extracts were compared to the peak area of the internal standard, n-tetratriacontane. The ratios of each of the four component peak areas to internal standard peak area measured during the equilibration test remained constant after 21 hours of mixing, indicating this was sufficient time to reach maximum solubility for those hydrocarbons with affinity to partition to the aqueous phase. This normalization of peak area was also used to confirm stability of the WAF during the acute range finder and definitive toxicity tests.

Test substance : 13% Kero, 12% LGO, 75% hydrotreated LVGO; sample ID A-2146
Reliability : (2) Reliable with restriction due to high pH value of purified water, i.e., values >8.5 for all treatments up to 9.88. However mortality was <10% for control treatments tested concurrently with each of the eight WAFS (5% was observed for one control treatment, no mortality for any other control tests). The high pH may not be of concern, given that the organisms are cultured using this purified water.

(24)

Type : Static
Species : Daphnia magna (Crustacea)
Exposure period : 48 hour(s)
Unit : mg/l
EL50 : 6.35 measured/nominal
Analytical monitoring : Yes
Method : OECD Guide-line 202
Year : 2000
GLP : Yes
Test substance : CAS No. 68476-34-6; diesel fuel (Gas oil, unspecified)

Method : Statistical Method: Probit analysis, Finney. D.J. Stat. Method in Biological Assay. 2nd ed. London, 1984.
Result : Number of immobilized daphnids after 48 hours were 0, 1, 10, 15, 15, and 17 in the control, 1.90, 3.75, 7.5, 15.0 & 30.0 mg/l treatments, respectively. 48-hr EL_{50} = 6.35 mg/l based upon nominal loading rate. 95% C.I. range 48-hr EL_{50} = 4.59-8.78 mg/l loading

Source	:	Study sponsor-DGMK, Deutsche Wissenschaftliche Gesellschaft fur Erdol, Erdgas und Kohle (German Research Organization for Oil, Gas and Coal). Distributed by CONCAWE.
Test condition	:	Test solutions were prepared as water accommodated fractions (WAFs). Control and dilution water were purified, sterilized drinking water. Purification includes filtration with charcoal, aeration and passage through a lime stone column. This final step may be a factor causing high, but apparently non-toxic pH values for the purified water (>8.5 to 9.88). Equilibration studies were conducted for a diesel and fuel oil at 100 and 1000 mg/l loading. WAFS were prepared in duplicate by mixing the appropriate mass of substance in 2 liters of water for 70 hr in sealed brown glass screw top bottles fitted w/ a port near the bottle bottom for drawing off WAF samples for GC analysis. An equilibration time of 21 hr was deemed sufficient to achieve maximum aqueous phase saturation of the gas oils. Range finding toxicity studies were conducted for all gas oils at 10 and 100 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Definitive toxicity studies were conducted at 1.90, 3.75, 7.5, 15.0 & 30.0 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Test vessels were sealed 200 ml conical flasks with 10 daphnids per flask and were completely filled with test solution. Dissolved oxygen was >60% saturation during the study. The pH of the test solutions ranged from 9.26 to 9.63 (high value may be concern). Temperature was 20.1-20.3 °C. Daphnia magna, STRAUS clone 5 organisms were supplied by testing laboratory; age < 24 hours old; second generation collected from cultures aged at least 3 weeks old. Analytical monitoring confirmed consistency of solubilized components of gas oil in aqueous solution, and was also used during WAF preparation to evaluate equilibration period required for maximum solubility of components. GC/FID analysis of cyclohexane extracts of WAF was based on ISO/DIS 9377-4:1999 method. The individual peak areas of four significant components detected in the WAF extracts were compared to the peak area of the internal standard, n-tetratriacontane. The ratios of each of the four component peak areas to internal standard peak area measured during the equilibration test remained constant after 21 hours of mixing, indicating this was sufficient time to reach maximum solubility for those hydrocarbons with affinity to partition to the aqueous phase. This normalization of peak area was also used to confirm stability of the WAF during the acute range finder and definitive toxicity tests.
Test substance	:	13% SR Kero CD3/4; 18% SR LGO CD3/4; 59% hydrotreated LGO CD3; 6% hydrotreated LGO CD4; 3% hydrotreated SGO CD3/4, 2% hydrotreated LCO; 2% hydrotreated Kero; sample ID A-2150
Reliability	:	(2) Reliable with restriction due to high pH value of purified water, i.e., values >8.5 for all treatments up to 9.88. However mortality was <10% for control treatments tested concurrently with each of the eight WAFS (5% was observed for one control treatment, no mortality for any other control tests). The high pH may not be of concern, given that the organisms are cultured using this purified water.

(24)

Type	:	Static
Species	:	Daphnia magna (Crustacea)
Exposure period	:	48 hour(s)
Unit	:	mg/l
EL50	:	14.49 measured/nominal
Analytical monitoring	:	Yes

Method	:	OECD Guide-line 202
Year	:	2000
GLP	:	Yes
Test substance	:	CAS No. 68476-30-2; fuel oil (Gas oil, unspecified)
Method	:	Statistical Method: Probit analysis, Finney. D.J. Stat. Method in Biological Assay. 2nd ed. London, 1984.
Result	:	Number of immobilized daphnids after 48 hours were 0, 0, 15, 15, 16 and 18 in the control, 1.5, 3.0, 6.00, 12.0 & 24.00 mg/l treatments, respectively. 48-hr EL_{50} = 14.49 mg/l based upon nominal loading rate.95% C.I. range 48-hr EL_{50} = 10.57-19.86 mg/l loading
Source	:	Study sponsor-DGMK, Deutsche Wissenschaftliche Gesellschaft fur Erdol, Erdgas und Kohle (German Research Organization for Oil, Gas and Coal). Distributed by CONCAWE.
Test condition	:	Test solutions were prepared as water accommodated fractions (WAFs). Control and dilution water were purified, sterilized drinking water. Purification includes filtration with charcoal, aeration and passage through a lime stone column. This final step may be a factor causing high, but apparently non-toxic pH values for the purified water (>8.5 to 9.88). Equilibration studies were conducted for a diesel and fuel oil at 100 and 1000 mg/l loading. WAFS were prepared in duplicate by mixing the appropriate mass of substance in 2 liters of water for 70 hr in sealed brown glass screw top bottles fitted w/ a port near the bottle bottom for drawing off WAF samples for GC analysis. An equilibration time of 21 hr was deemed sufficient to achieve maximum aqueous phase saturation of the gas oils. Range finding toxicity studies were conducted for all gas oils at 10 and 100 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Definitive toxicity studies were conducted at 1.5, 3.0, 6.00, 12.0, & 24.00 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Test vessels were sealed 200 ml conical flasks with 10 daphnids per flask and were completely filled with test solution. Dissolved oxygen was >60% saturation during the study. The pH of the test system solutions ranged from 9.15 to 9.25 (high value may be a concern). Temperature was 19.9-20.2 °C. Daphnia magna, STRAUS clone 5 organisms were supplied by testing laboratory; age < 24 hours old; second generation collected from cultures aged at least 3 weeks old. Analytical monitoring confirmed consistency of solubilized components of gas oil in aqueous solution, and was also used during WAF preparation to evaluate equilibration period required for maximum solubility of components. GC/FID analysis of cyclohexane extracts of WAF was based on ISO/DIS 9377-4:1999 method. The individual peak areas of four significant components detected in the WAF extracts were compared to the peak area of the internal standard, n-tetratriacontane. The ratios of each of the four component peak areas to internal standard peak area measured during the equilibration test remained constant after 21 hours of mixing, indicating this was sufficient time to reach maximum solubility for those hydrocarbons with affinity to partition to the aqueous phase. This normalization of peak area was also used to confirm stability of the WAF during the acute range finder and definitive toxicity tests
Test substance	:	36% hydrotreated LGO CD4; 16% hydrotreated LCO; 2% hydrotreated VGO; 3% hydrotreated VB-GO; 7% hydrotreated CC Kero; 6% SR Kero CD3/4; 23% SR LGO CD3/4; 1% LCO; 6% SRHGO; sample ID A-2151
Reliability	:	(2) Reliable with restriction due to high pH value of purified water, i.e., values >8.5 for all treatments up to 9.88. However mortality was <10% for control treatments tested concurrently with each of the eight WAFS (5%

was observed for one control treatment, no mortality for any other control tests). The high pH may not be of concern, given that the organisms are cultured using this purified water.

(24)

Type	:	Static
Species	:	Daphnia magna (Crustacea)
Exposure period	:	48 hour(s)
Unit	:	mg/l
EL50	:	36.01 measured/nominal
Analytical monitoring	:	Yes
Method	:	OECD Guide-line 202
Year	:	2000
GLP	:	Yes
Test substance	:	CAS No. 68476-34-6; No. 2 diesel fuel (Gas oil, unspecified)
Method	:	Statistical Method: Probit analysis, Finney. D.J. Stat. Method in Biological Assay. 2nd ed. London, 1984.
Result	:	Number of immobilized daphnids after 48 hours were 0, 0, 4, 5, 12, and 17 in the control, 6.25, 12.5, 25, 50 & 100 mg/l treatments, respectively. 48-hr EL_{50} = 36.01 mg/L based upon nominal loading rate.;95% C.I. range 48-hr EL_{50} = 25.52-50.79 mg/l loading
Source	:	Study sponsor-DGMK, Deutsche Wissenschaftliche Gesellschaft fur Erdol, Erdgas und Kohle (German Research Organization for Oil, Gas and Coal). Distributed by CONCAWE.
Test condition	:	Test solutions were prepared as water accommodated fractions (WAFs). Control and dilution water were purified, sterilized drinking water. Purification includes filtration with charcoal, aeration and passage through a lime stone column. This final step may be a factor causing high, but apparently non-toxic pH values for the purified water (>8.5 to 9.88). Equilibration studies were conducted for a diesel and fuel oil at 100 and 1000 mg/l loading. WAFS were prepared in duplicate by mixing the appropriate mass of substance in 2 liters of water for 70 hr in sealed brown glass screw top bottles fitted w/ a port near the bottle bottom for drawing off WAF samples for GC analysis. An equilibration time of 21 hr was deemed sufficient to achieve maximum aqueous phase saturation of the gas oils. Range finding toxicity studies were conducted for all gas oils at 10 and 100 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Definitive toxicity studies were conducted at 6.25, 12.5, 25, 50 & 100 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Test vessels were sealed 200 ml conical flasks with10 daphnids per flask and were completely filled with test solution. Dissolved oxygen was >60% saturation during the study. The pH of the test system solutions ranged from 9.55 to 9.65 (high value may be a concern). Temperature was 20.0-20.6 °C. Daphnia magna, STRAUS clone 5 organisms were supplied by testing laboratory; age < 24 hours old; second generation collected from cultures aged at least 3 weeks old. Analytical monitoring confirmed consistency of solubilized components of gas oil in aqueous solution, and was also used during WAF preparation to evaluate equilibration period required for maximum solubility of components. GC/FID analysis of cyclohexane extracts of WAF was based on ISO/DIS 9377-4:1999 method. The individual peak areas of four significant components detected in the WAF extracts were compared to the peak area of the internal standard, n-tetratriacontane. The ratios of each of the four component peak areas to internal standard peak area measured

during the equilibration test remained constant after 21 hours of mixing, indicating this was sufficient time to reach maximum solubility for those hydrocarbons with affinity to partition to the aqueous phase. This normalization of peak area was also used to confirm stability of the WAF during the acute range finder and definitive toxicity tests.

Test substance : DK-blend: middle range distillate; 81% hydrotreating, 19% straight run, sample ID A-2152

Reliability : (2) Reliable with restriction due to high pH value of purified water, i.e., values >8.5 for all treatments up to 9.88. However mortality was <10% for control treatments tested concurrently with each of the eight WAFS (5% was observed for one control treatment, no mortality for any other control tests). The high pH may not be of concern, given that the organisms are cultured using this purified water.

(24)

Type : Static
Species : Daphnia magna (Crustacea)
Exposure period : 48 hour(s)
Unit : mg/l
EL50 : 9.57 measured/nominal
Analytical monitoring : Yes
Method : OECD Guide-line 202
Year : 2000
GLP : Yes
Test substance : CAS No. 68476-34-6; diesel fuel (Gas oil, unspecified)

Method : Statistical Method: Probit analysis, Finney. D.J. Stat. Method in Biological Assay. 2nd ed. London, 1984.

Result : Number of immobilized daphnids after 48 hours were 0, 0, 9, 13, 18, and 19 in the control, 3.125, 6.25, 12.5, 25, and 50 mg/l treatments, respectively. Twenty daphnids per treatment were used.
48-hr EL_{50} = 9.57 mg/l based upon nominal loading rate. 95% C.I. range 48-hr EL_{50} = 7.34-12.46 mg/l loading.

Source : Study sponsor-DGMK, Deutsche Wissenschaftliche Gesellschaft fur Erdol, Erdgas und Kohle (German Research Organization for Oil, Gas and Coal). Distributed by CONCAWE.

Test condition : Test solutions were prepared as water accommodated fractions (WAF). Control and dilution water were purified, sterilized drinking water. Purification includes filtration with charcoal, aeration and passage through a lime stone column. This final step may be a factor causing high, but apparently non-toxic pH values for the purified water (>8.5 to 9.88). Equilibration studies were conducted for a diesel and fuel oil at 100 and 1000 mg/l loading. WAFS were prepared in duplicate by mixing the appropriate mass of substance in 2 liters of water for 70 hr in sealed brown glass screw top bottles fitted w/ a port near the bottle bottom for drawing off WAF samples for GC analysis. An equilibration time of 21 hr was deemed sufficient to achieve maximum aqueous phase saturation of the gas oils. Range finding toxicity studies were conducted for all gas oils at 10 and 100 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Definitive toxicity studies were conducted at 3.125, 6.25, 12.5, 25, & 50 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Test vessels were sealed 200 ml conical flasks with 10 daphnids per flask and were completely filled with test solution. Dissolved oxygen was >60% saturation during the study. The pH of the test system solutions ranged from 9.41 to 9.67 (high value may be a concern). Temperature was 20.1-20.3 °C. Daphnia magna, STRAUS clone 5

organisms were supplied by testing laboratory; age < 24 hours old; second generation collected from cultures aged at least 3 weeks old.

Analytical monitoring confirmed consistency of solubilized components of gas oil in aqueous solution, and was also used during WAF preparation to evaluate equilibration period required for maximum solubility of components. GC/FID analysis of cyclohexane extracts of WAF was based on ISO/DIS 9377-4:1999 method. The individual peak areas of four significant components detected in the WAF extracts were compared to the peak area of the internal standard, n-tetratriacontane. The ratios of each of the four component peak areas to internal standard peak area measured during the equilibration test remained constant after 21 hours of mixing, indicating this was sufficient time to reach maximum solubility for those hydrocarbons with affinity to partition to the aqueous phase. This normalization of peak area was also used to confirm stability of the WAF during the acute range finder and definitive toxicity tests.

Test substance : 31% Kerosene SR; 27% kerosene HDS; 21% middle oil HDS; 21% middle oil HC; sample ID A-2181

Reliability : (2) Reliable with restriction due to high pH value of purified water, i.e., values >8.5 for all treatments up to 9.88. However mortality was <10% for control treatments tested concurrently with each of the eight WAFS (5% was observed for one control treatment, no mortality for any other control tests). The high pH may not be of concern, given that the organisms are cultured using this purified water.

(24)

Type : Static
Species : Daphnia magna (Crustacea)
Exposure period : 48 hour(s)
Unit : mg/l
EL50 : 42.43 measured/nominal
Analytical monitoring : Yes
Method : OECD Guide-line 202
Year : 2000
GLP : yes
Test substance : CAS No. 68476-30-2; fuel oil (Gas oil, unspecified)

Method : Statistical Method: Probit analysis, Finney. D.J. Stat. Method in Biological Assay. 2nd ed. London, 1984.

Result : Number of immobilized daphnids after 48 hours were 0, 0, 3, 6, 14, and 17 in the control, 7.5, 15, 30, 60, and 120 mg/l treatments, respectively. Twenty daphnids per treatment were used.
48-hr EL_{50} = 42.43 mg/l based upon nominal loading rate, 95% C.I. range 48-hr EL_{50} = 31.59-56.97 mg/l loading.

Source : Study sponsor-DGMK, Deutsche Wissenschaftliche Gesellschaft fur Erdol, Erdgas und Kohle (German Research Organization for Oil, Gas and Coal). Distributed by CONCAWE.

Test condition : Test solutions were prepared as water accommodated fractions (WAFs). Control and dilution water were purified, sterilized drinking water. Purification includes filtration with charcoal, aeration and passage through a lime stone column. This final step may be a factor causing high, but apparently non-toxic pH values for the purified water (>8.5 to 9.88). Equilibration studies were conducted for a diesel and fuel oil at 100 and 1000 mg/l loading. WAFS were prepared in duplicate by mixing the appropriate mass of substance in 2 liters of water for 70 hr in sealed brown glass screw top bottles fitted w/ a port near the bottle bottom for drawing off

WAF samples for GC analysis. An equilibration time of 21 hr was deemed sufficient to achieve maximum aqueous phase saturation of the gas oils. Range finding toxicity studies were conducted for all gas oils at 10 and 100 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Definitive toxicity studies were conducted at 7.5, 15, 30, 60, & 120 mg/l loading, using WAFS which were divided into duplicate aliquots and tested. Test vessels were sealed 200 ml conical flasks with10 daphnids per flask and were completely filled with test solution. Dissolved oxygen was >60% saturation during the study. The pH of the test system solutions ranged from 9.17 to 9.33. Temperature was 19.9-20.2 °C. Daphnia magna, STRAUS clone 5 organisms were supplied by testing laboratory; age < 24 hours old; second generation collected from cultures aged at least 3 weeks old.

Analytical monitoring confirmed consistency of solubilized components of gas oil in aqueous solution, and was also used during WAF preparation to evaluate equilibration period required for maximum solubility of components. GC/FID analysis of cyclohexane extracts of WAF was based on ISO/DIS 9377-4:1999 method. The individual peak areas of four significant components detected in the WAF extracts were compared to the peak area of the internal standard, n-tetratriacontane. The ratios of each of the four component peak areas to internal standard peak area measured during the equilibration test remained constant after 21 hours of mixing, indicating this was sufficient time to reach maximum solubility for those hydrocarbons with affinity to partition to the aqueous phase. This normalization of peak area was also used to confirm stability of the WAF during the acute range finder and definitive toxicity tests.

Test substance : 7.4% Kerosene SR; 87% middle oil HDS; 5.7% middle oil SR; sample ID A-2182

Reliability : (2) Reliable with restriction due to high pH value of purified water, i.e., values >8.5 for all treatments up to 9.88. However mortality was <10% for control treatments tested concurrently with each of the eight WAFS (5% was observed for one control treatment, no mortality for any other control tests). The high pH may not be of concern, given that the organisms are cultured using this purified water.

(24)

Type : Static
Species : Daphnia magna (Crustacea)
Exposure period : 48 hour(s)
Unit : mg/l
LL50 : 2 measured/nominal
Analytical monitoring : Yes
Method : OECD Guide-line 202
Year : 1998
GLP : No
Test substance : CAS No. 68476-30-2; No. 2 fuel oil

Method : Statistical Method: Trimmed Spearman Karber Method, 1977.
Remark : Data have been developed which allow the quantification of complex hydrocarbons in water on a molar basis using SPME. (Parkerton, T.F., et al., Biomimetic Extraction as a Cost-Effective Analytical Tool for Determining the Aquatic Toxicity Hazard of Complex Petroleum Products, SETAC Europe, 2001). The measured molar concentrations reflect those portions that are potentially bioaccumulative in aquatic and terrestrial species. SPME is a surrogate for organism lipid and since it is measuring

dissolved, unbound hydrocarbon, the BPH concentration on the fiber is a direct measure of potential toxicity. Data developed at EMBSI comparing SPME molar concentrations of fuel oil in WAFs versus fuel oil toxicity assessed with a wide variety of aquatic species indicates that SPME quantification of bioavailable petroleum hydrocarbons (BPH) in fuel oil correlates well with observed aquatic toxicity. The total molar sum of components that partition to the fiber from the aqueous phase of a complex mixture in a lethal loading test "mimics" the total body residue in an aquatic test organism. Acute toxicity is predicted once a critical threshold on the SPME fiber (Cfiber,critical, also BPH critical) is exceeded. Thus, the Cfiber, critical provides a simple analytical measure that is comparable to the narcosis-based critical body residue (CBR) for a given test organism/endpoint.

Result :

Nominal Conc. (mg/l)	**BPH nmoles/mgC)**	**% Mortality 24hr**	**48 hr**
Control	2.9	0	0
1.0	42	0	5
1.5	65.2	5	45
3.5	105	20	90
11	144	35	100
28	162	65	100

Based on nominal loading rates:
48hr LL_{50}= 2.0 mg/;l; 95% confidence interval, 1.7-2.5 mg/l,
CBR=Cfiber,critical = 31 µmol/mlPDMS;
BPH critical = 85.3 nmol/mg C

Test condition : Nominal loading rates of 0, 1.0, 1.5, 3.5, 11 and 28.0 mg/l were used to prepare test solutions. Test solutions were prepared as water accommodated fractions (WAF). Individual treatments at each loading rate were prepared. Each treatment was prepared by adding the appropriate amount of test substance (volumetrically using a syringe) to ~2 liters (allowing for optimum surface contact between the test substance and the dilution water) of dilution water in a 2 liter size glass aspirator bottle. Syringe weights were recorded to determine actual loading rates. The solutions were mixed at a vortex of <10% of the static liquid depth of solution. The aspirator bottles were mixed in the dark (covered with dark plastic) for 24 hours on a magnetic stir plate with a Teflon® coated stir bar. After mixing, the treatments were allowed to settle for 1 hour. After settling, the water accommodated fraction (WAF) was removed and added to the test chambers. The control and dilution water reconstituted laboratory blend water prepared from carbon filtered well water and ion exchange softened, reverse osmosis dialyzed well water aged for >24 hrs.. BPH analysis was performed using SPME (solid phase microextraction) fibers which had been equilibrated overnight in WAF solutions and quantified using gas chromatography equipped with a flame ionization detector. Daphnia magna were supplied by testing laboratory; age < 24 hours old; second generation collected from cultures aged sixteen days old. Four replicates per treatment and 5 organisms per replicate were tested for each treatment and the control. Test vessels were 125 ml autoclaved flasks filled with no headspace and tightly sealed to prevent volatilization. Water temperature range was 20.3 to 21.6 °C. Test photoperiod was 16 hrs. light and 8 hr dark, light intensity approx 69 foot-candles during full daylight periods. Dissolved oxygen measurements ranged from 7.4 to 8.0 ppm, pH values between 7.5 and 7.8. Duplicate samples were taken of the control on Day 0 and each WAF (from the mixing vessel). Samples were used to determine bioavailable petroleum hydrocarbons (BPH). Measurement of dissolved, bioavailable hydrocarbon was performed using solid phase

microextraction (SPME) fiber analysis by GC/FID. BPH samples were mixed (minimal headspace) in vessels containing the SPME fibers overnight. Analyses were performed using 100 µm PDMS fibers obtained from Supelco. 125 ml glass flasks fitted with ground glass stoppers and equipped with stir bars were filled with WAF samples. SPME fibers were inserted through a small hole drilled in the center of the glass stopper. The total moles of hydrocarbons that partition to the fibers were quantified using a gas chromatograph equipped with a flame ionization detector (GC-FID). FID detector response was translated into molar hydrocarbon concentration (BPH as nmoles/mg C) using the molar response based on a number of aromatic standards. BPH values are reported as nanomoles of quantified hydrocarbon per mg of carbon based on carbon content of polydimethylsiloxane (PDMS) fiber coating. Critical SPME fiber concentration (Cfiber, critical), comparable to critical total body residue (CBR, on a lipid basis) is calculated from BPH results and is reported in units of µmoles/ml of PDMS. Quantitation was based on 2, 3 dimethyl napthalene since a wide variety of hydrocarbons exhibited a relative molar response of within a factor of two when normalized to this compound. Calculation of CBR as Cfiber, critical is based on Verbruggen et al.

Reliability : (2) Reliable with restrictions.
The data reported in this report were obtained as part of a research program to correlate toxicity with Bioavailable Petroleum Hydrocarbons (BPH). Thus although the work was conducted according to relevant test guidelines and standard operating procedures, the research was not done in strict accordance with GLPs. All data reported as final were audited by EBSI Quality Assurance unit.

(23) (39)

Type : Static
Species : Daphnia magna (Crustacea)
Exposure period : 48 hour(s)
Unit : mg/l
EL50 : 210 measured/nominal
Analytical monitoring : Yes
Method : OECD Guide-line 202
Year : 1995
GLP : Yes
Test substance : CAS No. 68334-30-5; Gas oil

Method : Statistical Method: Probit analysis
Result : 48-hr EL_{50} = 210 mg/l
95% confidence interval of 160 - 270 mg/l based on nominal loading rates. Numbers of immobilized daphnids after 48 hrs were 0, 0, 0, 0, 5, 11, 15, and 20 in the 0, 10, 22, 46, 100, 220, 460, and 1000 mg/l treatments. No excursions from protocol were noted. Analytical method used was gas chromatography-mass spectrometry. Mean reduction in the concentration of dissolved hydrocarbons during the test was 5.5% (range 0 - 23%).

Test condition : Individual treatment concentrations were prepared as water accommodated fractions (WAF). Nominal loading rates in the definitive test were 0, 10, 22, 46, 100, 220, 460, and 1000 mg/l. Control and dilution water was reconstituted hard water prepared by adding salts to reverse osmosis filtered water following EPA guidelines (hardness 190 mg/l as $CaCO_3$).
Test substance was mixed in dilution water for 68 hrs. Mixing time was determined in an equilibration study in which the test substance concentration in the aqueous phase of the WAFs was monitored by GC-

MS. Mixtures were allowed to settle 1.5 hrs prior to drawing off the aqueous phase for testing. Test vessels were sealed 150-ml Erlenmeyer flasks with 10 daphnids per vessel. Test daphnids were <24 hrs old and collected from cultures supplied by the testing laboratory that have been aged between 14 and 28 days. Two replicates per treatment and control were used. Test temperature was 18.0 - 18.6 °C. Photoperiod was 16 hrs light and 8 hrs dark. Dissolved oxygen was >60% saturation (9.0 to 9.3 mg/l). pH was 7.6 - 8.4. Samples were collected at the beginning and end of the test to monitor the concentration of soluble components in the test solutions.

Reliability : (1) valid without restriction

(34)

Type : Static
Species : Daphnia magna (Crustacea)
Exposure period : 48 hour(s)
Unit : mg/l
EL50 : 68 measured/nominal
Analytical monitoring : Yes
Method : OECD Guide-line 202
Year : 1995
GLP : Yes
Test substance : CAS No. 68334-30-5; Gas oil

Method : Statistical Method: Moving average angle method
Result : 48-hr EL_{50} = 68 mg/l 95% confidence interval of 49 - 94 mg/l based on nominal loading rates.
Numbers of immobilized daphnids after 48 hrs were 0, 0, 0, 0, 20, 20, 20, and 20 in the 0, 10, 22, 46, 100, 220, 460, and 1000 mg/l treatments.
No excursions from protocol were noted. Analytical method used was gas chromatography-mass spectrometry. Mean reduction in the concentration of dissolved hydrocarbons during the test was 15% (range 0 - 23%).

Test condition : Individual treatment concentrations were prepared as water accommodated fractions (WAF). Nominal loading rates in the definitive test were 0, 10, 22, 46, 100, 220, 460, and 1000 mg/l. Control and dilution water was reconstituted hard water prepared by adding salts to reverse osmosis filtered water following EPA guidelines (hardness 162 mg/l as $CaCO_3$).
Test substance was mixed in dilution water for 48 hrs in sealed vessels with minimal headspace. Mixing time was determined in an equilibration study in which the test substance concentration in the aqueous phase of the WAFs was monitored by GC-MS. Mixtures were allowed to settle 1.5 hrs prior to drawing off the aqueous phase for testing. Test vessels were sealed 150-ml Erlenmeyer flasks with 10 daphnids per vessel. Test daphnids were <24 hrs old and collected from cultures supplied by the testing laboratory that have been aged between 14 and 28 days. Two replicates per treatment and control were used. Test temperature was 18.1 - 18.9 °C. Photoperiod was 16 hrs light and 8 hrs dark. Dissolved oxygen was >60% saturation (9.1 to 9.2 mg/l). pH was 8.2 - 8.4. Samples were collected at the beginning and end of the test to monitor the concentration of soluble components in the test solutions.

Reliability : (2) valid with restrictions

(35)

Type : Static
Species : Daphnia magna (Crustacea)
Exposure period : 48 hour(s)

Unit	:	mg/l
EL50	:	13 measured/nominal
Analytical monitoring	:	Yes
Method	:	OECD Guide-line 202
Year	:	1994
GLP	:	No
Test substance	:	CAS No. 68334-30-5; Diesel (AGO)
Method	:	Statistical Method: Moving average angle method
Result	:	48-hr EL_{50} = 13 mg/l 95% confidence interval of 11 - 15 mg/l based on nominal loading rates. Numbers of immobilized daphnids after 48 hrs were 0, 0, 0, 0, 0, 2, 20, and 20 in the 0, 0.1, 0.3, 1, 3, 10, 30, and 100 mg/l treatments.
Test condition	:	Individual treatment concentrations were prepared as water accommodated fractions (WAF). Nominal loading rates in the definitive test were 0, 0.1, 0.3, 1, 3, 10, 30, and 100 mg/l. Control and dilution water was reconstituted hard water prepared by adding salts to reverse osmosis water following EPA guidelines (hardness 180 mg/l as $CaCO_3$). Test substance was mixed in dilution water for 24 hrs in sealed vessels with minimal headspace. Mixtures were allowed to settle 1 hr prior to drawing off the aqueous phase for testing. Test vessels were sealed 140-ml Erlenmeyer flasks (no headspace) with 10 daphnids per vessel. Test daphnids were obtained from the third brood onwards of cultures supplied by the testing laboratory that have been aged <28 days. Two replicates per treatment and control were used. Test temperature was 17.0 - 19.0 °C. Photoperiod was 16 hrs light and 8 hrs dark. Dissolved oxygen was 9.2 to 9.8 mg/l). pH was 7.9 - 8.1. Samples were collected from the WAFs at the beginning of the test for BTEX analysis by purge and trap GC-FID.
Reliability	:	(2) Reliable with restrictions. Test methods were sufficiently documented and EPA methods were referenced, even though no test guidelines were specified and the test was not conducted under GLP. Chemical analyses conducted were not aimed at monitoring test substance concentrations in the aqueous phase of the WAFs during exposure.

(31)

Type	:	Static
Species	:	Daphnia magna (Crustacea)
Exposure period	:	48 hour(s)
Unit	:	mg/l
Analytical monitoring	:	No
Method	:	OECD Guide-line 202
Year	:	2003
GLP	:	No data
Test substance	:	Diesel fuel CAS No. 68334-30-5
Remark	:	Details on the number of exposure levels, concentrations used and any water chemistry measurements made during the test were not provided in the reviewed report. However, the methods of exposure solution preparation and testing (sealed vessels) provide a high level of confidence that the results give a reliable assessment of the toxicity of the test substance to Daphnia magna.
Result	:	48-h EL_{50} = 100 - 300 mg/l No Observed Effect Level (NOEL) = 100 mg/l There was no control mortality during the test.

Test condition	:	Exposure solutions were prepared as water accommodated fractions (WAFs) and were expressed as the test substance loading rate per volume of dilution water (mg/l). WAFs were prepared by adding measured amounts of diesel fuel to measured volumes of dilution water. Two-liter glass flasks containing 2.3 l of dilution water and diesel fuel were sealed, leaving only a small amount of headspace. The contents then were stirred at 150 rpm for approximately 72 hours (±2 hours). After stirring, the contents were allowed to settle for 1-2 hours to permit undissolved material to separate from the WAF. The aqueous phase then was drawn off for use in the test. Control water was subjected to the same preparation regime except no test substance was added to the flasks. Dilution water used in the test was reconstituted fresh water. Test vessels were 150 ml glass Erlenmeyer flasks that were completely filled with the WAF solution. Duplicate flasks were prepared for each WAF treatment and control group. Ten daphnids were added to each flask and the flasks were sealed with a screw cap. The numbers of immobilized daphnids were determined at 24 and 48 hours. Daphnids were considered immobile if they were not observed to swim during a 15 second observation period.
Reliability	:	(2) valid with restrictions Details of the testing methods and results of any water chemistry measurements were not provided in the report. Also, there were no analytical measurements made on the WAF solutions.

(12)

4.3 TOXICITY TO AQUATIC PLANTS E.G. ALGAE

Species	:	Selenastrum capricornutum (Algae)
Exposure period	:	72 hour(s)
Unit	:	mg/l
Analytical monitoring	:	Yes
Method	:	OECD Guide-line 201 "Algae, Growth Inhibition Test"
Year	:	1998
GLP	:	No
Test substance	:	CAS No. 68476-30-2; Fuel Oil, No. 2
Method	:	Statistical Method: ELb50 values for cell density were determined using linear interpolation method according to the OECD 201 method. The ELr_{50} values for growth rate were calculated based on linear regression of the ln cell density vs time using PROC REGRESSION in SAS.
Remark	:	Data have been developed which allow the quantification of complex hydrocarbons in water on a molar basis using SPME. (Parkerton, T.F., et al., Biomimetic Extraction as a Cost-Effective Analytical Tool for Determining the Aquatic Toxicity Hazard of Complex Petroleum Products SETAC Europe, 2001). The measured molar concentrations reflect those portions that are potentially bioaccumulative in aquatic and terrestrial species. SPME is a surrogate for organism lipid and since it is measuring dissolved, unbound hydrocarbon, the BPH concentration on the fiber is a direct measure of potential toxicity. Data developed at EMBSI comparing SPME molar concentrations of fuel oil in WAFs versus fuel oil toxicity assessed with a wide variety of aquatic species indicates that SPME quantification of bioavailable petroleum hydrocarbons (BPH) in fuel oil correlates well with observed aquatic toxicity. The total molar sum of components that partition to the fiber from the aqueous phase of a complex

mixture in a lethal loading test "mimics" the total body residue in an aquatic test organism. Acute toxicity is predicted once a critical threshold on the SPME fiber (Cfiber,critical, also BPH critical) is exceeded. Thus, the Cfiber, critical provides a simple analytical measure that is comparable to the narcosis-based critical body residue (CBR) for a given test organism/endpoint.

Result :

Nominal Conc. (mg/l)	BPH (nmoles/mgC)	72 h % Inhibition		
		Cell density	Cell Growth	Growth Rate
Control	----------not applicable----------			
0.4	23.9	3.4	2.6	0.4
0.8	26.	-3.4	-2.2	-2.2
1.6	57	48	37	11
3.2	146.7	92	94	66
Based on nominal loading rates:				
72-hr EbL_{50} (mg/l)		1.8	1.9	2.9
95% confidence interval		1.6-2.1	1.5-2.4	could not calculate

CBR =Cfiber, critical = 23 µmol/mlPDMS;
BPH critical = 63 nmol/mg
CNOEC-not calculated

Test condition : Control and dilution water was algal nutrient media (preparation and composition referenced in EPA-600/9-78-018). Nominal loading rates in the definitive test were 0, 0.4, 0.8, 1.6, and 3.2 mg/l. Individual treatment concentrations were prepared as water accommodated fractions (WAF) at each loading rate and used for toxicity testing and GC analysis. Each treatment was prepared by adding the appropriate amount of test substance (volumetrically using a syringe) to 4 liters (allowing for optimum surface contact between the test substance and the dilution water) of dilution water in a 4 liter size glass aspirator bottle. Syringe weights were recorded to determine actual loading rates. The solutions were mixed at a vortex of 20% of the static liquid depth of solution. The treatments were mixed for 24 hours on a magnetic stir plate with a Teflon® coated stir bar. After mixing, the treatments were allowed to settle for 2 hours. After settling, the water accommodated fraction (WAF) was removed from the outlet at the bottom of the vessel. An aliquot of each treatment was removed for pH measurement and SPME extraction. Test vessels were sealed 125 ml Erlenmeyer flasks containing 10 glass beads and filled with test solution. There were five flasks for each test and control treatment. Algal cells were obtained from in-house cultures which were 5 days old at the start of the test. All flasks were inoculated with algal cells to yield an initial concentration of 5000 cells/ml. The appropriate amount of algae was added to each test chamber then the test solutions were added. Once test chambers were filled they were sealed immediately. Test chambers were only opened to remove a sample for cell density determination on Days 1-3. The volume of test solution removed for each evaluation was replaced with spare solution that had been stored in a sealed vessel. A separate spare vessel was prepared for each replacement interval. Test flasks containing glass beads were conditioned by rinsing with the appropriate solution. Test flasks were hand shaken once or twice daily to ensure that the algal cells remain in suspension. Each test flask was placed on a shaker table (100 rpm) for the duration of the study. Cell counts on each test flask were performed daily. Test temperature was 24.8 °C, 0.1° S.D. Test photoperiod was 14 hrs of light, ten hrs dark, with light intensity ranging from 4100 to 4200 Lux. Direct cell counts on each test flask were performed daily using a hemocytometer. Duplicate samples were taken of the control on Day 0 and each WAF (from the mixing vessel) and from a

composite of replicates 1 through 5 at termination. Samples were used to determine bioavailable petroleum hydrocarbons (BPH). Measurement of dissolved, bioavailable hydrocarbon was performed using solid phase microextraction (SPME) fiber analysis by GC/FID. BPH samples were mixed (minimal headspace) in vessels containing the SPME fibers overnight.
Analyses were performed using 100 um PDMS fibers obtained from Supleco. 125 ml glass flasks fitted with ground glass stoppers and equipped with stir bars were filled with WAF samples. SPME fibers were inserted through a small hole drilled in the center of the glass stopper. The total moles of hydrocarbons that partition to the fibers were quantified using a gas chromatograph equipped with a flame ionization detector (GC-FID). FID detector response was translated into molar hydrocarbon concentration (BPH as nmoles/mg C) using the molar response based on a number of aromatic standards.
BPH values are reported as nanomoles of quantified hydrocarbon per mg of carbon based on carbon content of polydimethylsiloxane (PDMS) fiber coating. Critical SPME fiber concentration (Cfiber, critical), comparable to critical total body residue (CBR, on a lipid basis) is calculated from BPH results and is reported in units of µmoles/ml of PDMS. Quantitation was based on 2, 3 dimethyl napthalene since a wide variety of hydrocarbons exhibited a relative molar response of within a factor of two when normalized to this compound. Calculation of CBR as Cfiber, critical is based on Verbruggen et al.

Reliability : (2) Reliable with restriction.
The study data in this report were developed as part of a research program to correlate BPH with toxicity. Thus although the work was conducted according to the relevant guidelines and standard laboratory operating procedures (SOPs), the research work was not done in strict accordance with Good Laboratory Procedures. However, all data in the final report were audited by EBSI QA unit.

(17) (39)

Species : Selenastrum capricornutum (Algae)
Exposure period : 72 hour(s)
Unit : mg/l
Analytical monitoring : Yes
Method : OECD Guide-line 201 "Algae, Growth Inhibition Test"
Year : 1995
GLP : Yes
Test substance : CAS No. 68334-30-5; Gas oil

Method : Statistical Method: EL50 values determined by probit analysis. Williams test used to determine NOELs.

Result : Based on nominal loading rates:
72-hr EL_{50} (biomass) = 25 mg/l
95% confidence interval of 21 - 30 mg/l

72-hr EL_{50} (growth rate) = 78 mg/l
95% confidence interval of 64 - 96 mg/l

72-hr NOEL (biomass) = 3 mg/l
72-hr NOEL (growth rate) = 10 mg/l.

Nominal Conc. (mg/l)	72 h % Inhibition	72 h Mean Cell Conc. (million cells/ml)
Control	n/a	0.14
1.0	-21	0.17
3.0	-36	0.19
10	29	0.10
30	61	0.055
100	91	0.013
300	94	0.0087
1000	95	0.0074

n/a - Not applicable
The pH increased by more than one unit (1.1 units) during the test as a result of good culture growth and could not be avoided. Temperature range in the incubator was outside the preferred range of 21 - 25 °C. The out-of-range readings were recorded in the first four hours of the test probably due to temperature fluctuations associated with opening and closing the incubator. These deviations were not sufficient to invalidate the study. Analytical method used was gas chromatography-mass spectrometry. Mean reduction in the concentration of dissolved hydrocarbons during the test was 14% (range 0 - 40%).

Test condition : Individual treatment concentrations were prepared as water accommodated fractions (WAF). Nominal loading rates in the definitive test were 0, 1, 3, 10, 30, 100, 300, and 1000 mg/l. Control and dilution water was algal nutrient medium prepared according to EPA guidelines except that boric acid was present at 105 µg/l and sodium bicarbonate at 50 mg/l.
Test substance was mixed with dilution water for 72 hrs, and the mixture was allowed to settle for 1.5 - 2 hrs prior to drawing off the aqueous phase for testing. Test vessels were sealed 287 ml Erlenmeyer flasks filled with test solution. There were four flasks for each treatment and seven control flasks. Three of the four treatment and six of the seven control flasks were inoculated with algal cells to yield an initial concentration of 5000 cells/ml. Algal cells were obtained from laboratory cultures that were originally derived from a strain from American Type Culture Collection (ATCC 22662). Uninoculated flasks were used to determine particle counts without algal cells using a Coulter Multisizer. Flasks were incubated in a cooled orbital (100 cycles/min) incubator. Biomass was calculated as area under the growth curve. Test temperature was 19.5 - 27.6 °C. Lighting was continuous at ~5100 lux. The pH ranged from 8.3 - 8.7 at test initiation and 7.9 - 9.5 at test termination. Samples were collected at the beginning and end of the test to monitor the concentration of soluble components in the test solutions.

Reliability : (1) valid without restriction

(32)

Species : Selenastrum capricornutum (Algae)
Exposure period : 72 hour(s)
Unit : mg/l
Analytical monitoring : Yes
Method : OECD Guide-line 201 "Algae, Growth Inhibition Test"
Year : 1995
GLP : Yes
Test substance : CAS No. 68334-30-5; Gas oil

Method : Statistical Method: EL_{50} values determined by probit

analysis. Williams test used to determine NOELs.

Result : Based on nominal loading rates:
72-hr EL_{50} (biomass) = 10 mg/l 95% confidence interval of 8.4 - 12 mg/l

72-hr EL_{50} (growth rate) = 22 mg/l 95% confidence interval of 19 - 26 mg/l

72-hr NOEL (biomass) = 1 mg/l
72-hr NOEL (growth rate) = 3 mg/l.

Nominal Conc. (mg/l)	72 h % Inhibition	72 h Mean Cell Conc. (million cells/ml)
Control	n/a	0.17
1.0	0	0.17
3.0	24	0.13
10	53	0.08
30	91	0.016

n/a - Not applicable

Only four concentrations were tested which is less than a minimum of five concentrations stated in the guidelines.
Analytical method used was gas chromatography-mass spectrometry.
Mean reduction in the concentration of dissolved hydrocarbons during the test was 14% (range 5 - 25%).

Test condition : Individual treatment concentrations were prepared as water accommodated fractions (WAF). Nominal loading rates in the definitive test were 0, 1, 3, 10 and 30 mg/l. Control and dilution water was algal nutrient medium prepared according to EPA guidelines except that boric acid was present at 105 µg/l and sodium bicarbonate at 50 mg/l. Test substance was mixed with dilution water for ~48 hrs, and the mixture was allowed to settle for 1.5 hr prior to drawing off the aqueous phase for testing. Test vessels were sealed 287 ml Erlenmeyer flasks filled with test solution. There were four flasks for each treatment and seven control flasks. Three of the four treatment and six of the seven control flasks were inoculated with algal cells to yield an initial concentration of 5000 cells/ml. Algal cells were obtained from laboratory cultures that were originally derived from a strain from American Type Culture Collection (ATCC 22662). Uninoculated flasks were used to determine particle counts without algal cells using a Coulter Multisizer. Flasks were incubated in a cooled orbital (100 cycles/min) incubator. Biomass was calculated as area under the growth curve. Test temperature was 21.3 - 23.6 °C. Lighting was continuous at ~3720 lux. The pH ranged from 8.0 - 8.6 at test initiation and 8.5 - 9.2 at test termination. Samples were collected at concentration of soluble components in the test solutions.

Reliability : (1) valid without restriction

(33)

Species : Skeletonema costatum (Algae)
Exposure period : 72 hour(s)
Unit : mg/l
Analytical monitoring : Yes
Method : other: ASTM Annual Book of Standards, Volume 11.04, Standard Guide for Conducting Static 96 hour Toxicity Tests with Macroalgae, E1218-90
Year : 1995
GLP : No
Test substance : CAS No. 68476-30-2; Fuel Oil, No. 2

Method : Statistical Method: ELb_{50} values for cell density were determined using linear interpolation method according to the OECD 201 method. The ELr_{50} values for growth rate were calculated based on linear regression of the ln cell density vs time using PROC REGRESSION in SAS.

Remark : Data have been developed which allow the quantification of complex hydrocarbons in water on a molar basis using SPME. (Parkerton, T.F., et al., Biomimetic Extraction as a Cost-Effective Analytical Tool for Determining the Aquatic Toxicity Hazard of Complex Petroleum Products SETAC Europe, 2001). The measured molar concentrations reflect those portions that are potentially bioaccumulative in aquatic and terrestrial species. SPME is a surrogate for organism lipid and since it is measuring dissolved, unbound hydrocarbon, the BPH concentration on the fiber is a direct measure of potential toxicity. Data developed at EMBSI comparing SPME molar concentrations of fuel oil in WAFs versus fuel oil toxicity assessed with a wide variety of aquatic species indicates that SPME quantification of bioavailable petroleum hydrocarbons (BPH) in fuel oil correlates well with observed aquatic toxicity. The total molar sum of components that partition to the fiber from the aqueous phase of a complex mixture in a lethal loading test "mimics" the total body residue in an aquatic test organism. Acute toxicity is predicted once a critical threshold on the SPME fiber (Cfiber,critical, also BPH critical) is exceeded. Thus, the Cfiber, critical provides a simple analytical measure that is comparable to the narcosis-based critical body residue (CBR) for a given test organism/endpoint.

Result :

Nominal Conc. (mg/l)	BPH (nmoles/mgC)	72 h % Inhibition Cell density	Cell Growth	Growth Rate
Control	0.3	---not applicable----		-
0.4	15.1	-6.3	12	-0.67
1.0	30.4	-19	11	1.0
2.4	77.4	58	43	32
6.0	166.7	92	83	92
15	207.3	98	83	100
Based on nominal loading rates:				
72-hr EL_{50} (mg/l)		2.2	5.8	2.2
95% confidence interval		2.0-2.4	could not calculate	0.5-16.2

Test condition : Control and dilution water was enriched seawater nutrient medium with 100 mg/l sodium. Nominal loading rates in the definitive test were 0, 0.4, 1.0, 2.4, 6.0 and 15 mg/l.
Individual treatment concentrations were prepared as water accommodated fractions (WAF) at each loading rate and used for toxicity testing and GC analysis. Each treatment was prepared by adding the appropriate amount of test substance (volumetrically using a syringe) to 2 liters (allowing for optimum surface contact between the test substance and the dilution water) of dilution water in a 2 liter size glass aspirator bottle. Syringe weights were recorded to determine actual loading rates. The solutions were mixed at a vortex of 20% of the static liquid depth of solution. The treatments were mixed for 24 hours on a magnetic stir plate with a Teflon® coated stir bar. After mixing, the treatments were allowed to settle for 2 hours. After settling, the water accommodated fraction (WAF) was removed from the outlet at the bottom of the vessel. An aliquot of each treatment was removed for pH measurement and SPME extraction. Test vessels were sealed 125 ml Erlenmeyer flasks filled with test solution. There were four flasks for each test and control treatment. All flasks were inoculated with algal cells to yield an initial concentration of 10,000 cells/ml.

Algal cells were obtained from in-house cultures which were 5 days old at the start of the test. The appropriate amount of algae was added to each test chamber then the test solutions were added. Once test chambers were filled they were sealed immediately. Test chambers were only opened to remove a sample for cell density determination on Days 1-3. The volume of test solution removed for each evaluation was replaced with spare solution that had been stored in a sealed vessel. A separate spare vessel was prepared for each replacement interval.
Test flasks were hand shaken once or twice daily to ensure that the algal cells remained in suspension. Test temperature was 20.4 °C, 0.3° S.D. Test photoperiod was 14 hrs of light, ten hrs dark, with light intensity ranging from 4100 to 4200 Lux. Direct cell counts on each test flask were performed daily using a hemocytometer. Duplicate samples were taken of each WAF (from the mixing vessel) and the control on Day 0 and from a composite of replicates 1 through 5 at termination. Samples were used to determine bioavailable petroleum hydrocarbons (BPH). Measurement of dissolved, bioavailable hydrocarbon was performed using solid phase microextraction (SPME) fiber analysis by GC/FID. BPH samples were mixed (minimal headspace) in vessels containing the SPME fibers overnight. Analyses were performed using 100 µm PDMS fibers obtained from Supleco. 125 ml glass flasks fitted with ground glass stoppers and equipped with stir bars were filled with WAF samples. SPME fibers were inserted through a small hole drilled in the center of the glass stopper. The total moles of hydrocarbons that partition to the fibers were quantified using a gas chromatograph equipped with a flame ionization detector (GC-FID). FID detector response was translated into molar hydrocarbon concentration (BPH as nmoles/mg C) using the molar response based on a number of aromatic standards.
BPH values are reported as nanomoles of quantified hydrocarbon per mg of carbon based on carbon content of polydimethylsiloxane (PDMS) fiber coating. Critical SPME fiber concentration (Cfiber, critical), comparable to critical total body residue (CBR, on a lipid basis) is calculated from BPH results and is reported in units of µmoles/ml of PDMS. Quantitation was based on 2, 3 dimethyl napthalene since a wide variety of hydrocarbons exhibited a relative molar response of within a factor of two when normalized to this compound. Calculation of CBR as Cfiber, critical is based on Verbuggen et al.

Reliability : (2) Reliable with restriction.
The study data in this report were developed as part of a research program to correlate BPH with toxicity. Thus although the work was conducted according to the relevant guidelines and standard laboratory operating procedures (SOPs), the research work was not done in strict accordance with Good Laboratory Procedures. However, all data in the final report were audited by EBSI QA unit.

(18) (39)

Species : other algae: Raphidocelis subcapitata
Exposure period : 72 hour(s)
Unit : mg/l
Analytical monitoring : No
Method : OECD Guide-line 201 "Algae, Growth Inhibition Test"
Year : 2003
GLP : No data
Test substance : Diesel fuel CAS No. 68334-30-5

Remark : Details on the number of exposure levels, concentrations used and any

water chemistry measurements made during the test were not provided in the reviewed report. However, the methods of exposure solution preparation and testing (sealed vessels) provides a high level of confidence that the results give a reliable assessment of the toxicity of the test substance to R. subcapitata.

Result : 72-hour Area Under the Growth Curve
EbL_{50} = 10 - 22 mg/l
No Observed Effect Level = <1 mg/l

72-hour Average Specific Growth Rate
ErL_{50} = 22 - 46 mg/l
No Observed Effect Level = <1 mg/l

The growth in the control flasks exceeded a factor of 16 within the 3 days of the test and therefore achieved the validity criteria of the test guidelines.

Test condition : Exposure solutions were prepared as water accommodated fractions (WAFs) and were expressed as the test substance loading rate per volume of dilution water (mg/l). WAFs were prepared by adding measured amounts of diesel fuel to measured volumes of algal growth medium. Two-liter glass flasks containing 2.3 l of growth medium and diesel fuel were sealed, leaving only a small amount of headspace. The contents then were stirred at 150 rpm for approximately 72 hours (±2 hours). After stirring, the contents were allowed to settle for 1-2 hours to permit undissolved material to separate from the WAF. The aqueous phase then was drawn off for use in the test. Algal growth medium for the control group was subjected to the same preparation regime except no test substance was added to the flasks.

The sealed 72-h growth inhibition test was carried out in 287 ml full-volume Erlenmeyer flasks. To each flask was added 5.7 ml of a 2.5 g/l solution of sodium bicarbonate followed by the WAF solution or control medium to make up 287 ml. Four replicate flasks were used for each WAF treatment while seven replicate flasks were used for the control group. Three out of each set of four flasks containing WAF and six out of the seven control flasks were inoculated with sufficient R. subcapitata to give an initial concentration of approximately 5000 cells/ml. The uninoculated flasks were used to determine background particle counts in the absence of algal cells. The flasks were sealed and randomly placed in cooled orbital incubators (approximately 100 cycles/min at a nominal
23±2 °C) under constant illumination. Cell counts were made using a Coulter Multisizer on samples taken from each flask at the start of the test and then at approximately 24 h intervals. Results were evaluated using growth measures based on area under the growth curve and average specific growth rate as recommended in the OECD test guidelines. The subscripts EbL_{50} and ErL_{50} were used to differentiate between measures of effect on the basis of area under the growth curve and growth rate.

Reliability : (2) valid with restrictions
Details of the testing methods and results of any water chemistry measurements were not provided in the report. Also, there was no analytical measurements made on the WAF solutions.

(12)

5.1.1 ACUTE ORAL TOXICITY

Type	:	LD_{50}
Value	:	9 ml/kg bw
Species	:	Rat
Strain	:	Sprague-Dawley
Sex	:	Male/female
Number of animals	:	10
Vehicle	:	Undiluted
Year	:	1980
GLP	:	No data
Test substance	:	Diesel fuel, sample API 79-6 (See section 1.1.1.)

Method :

Food was withheld from the rats overnight prior to dosing.
A single dose of test material was given by gavage to groups of 5 male and 5 female rats at dose levels of 2.5, 5.0, 10, 15 & 20 ml/kg.
Daily observations were made for death or signs of toxicity during the 14 day duration of the study. Body weights were recorded at the start and on the 7th and 14th day of the study.
A gross necropsy was performed on all animals that died during the study and on all survivors that were sacrificed on day 14.

Result : Mortality rates were as follows:

Dose group (ml/kg)	Mortality (%)
2.5	12.5
5	20
10	70
15	40
20	90

Signs of toxicity were the same for all dose groups and increased in severity with increasing dose. The signs included oily urine stains and oily diarrhea. The urine and feces stayed on the fur and caused hair loss, irritation, redness and sores on the affected skin. In many animal open sores were observed on the skin surrounding the anus. Blood around the eyes, nose and mouth was also common. Other signs noted included lethargy and pus or blood at the urinary orifice. Observations at gross necropsy were similar for each dose group.
Almost all animals that died before the 14th day had intestinal damage. The intestines and often the stomach were hemorrhagic, sometimes observed with blood. The intestinal walls were thin. Test material was found in the cecum for many days after dosing and a few rats had white spots on their cecums and an increased amount of gas was noted in the intestinal tract.
Animals surviving 14 days had fewer abnormalities, all minor in nature. These included enlarged Peyer's patches on the intestine, an indication that some irritation had occurred.

Conclusion : The oral median lethal dose was 9.0 ml/kg with a 95% confidence interval of 5.58 to 14.51 ml/kg.

Reliability : (1) valid without restriction
Although the study was conducted before Good Laboratory Practices were published, the study appears to have been conducted using appropriate methods and to have been fully reported.

(7)

Type : LD50
Species : Rat
Test substance : Home heating oil containing cracked stocks (See section 1.1.1.)

Result : API reported acute oral toxicity studies on three samples of home heating oil prepared from blends of straight run and cracked stocks.
The LD_{50} values are shown below

Sample	Saturates content (%)	LD_{50} (ml/kg)	API report number
API 78-4	67.8	21.2	API 27-32068
API 78-2	73.4	19	API 27-32771
API 78-3	79.2	14.5	API 27-32773

Reliability : (1) valid without restriction
Although the studies were conducted before Good Laboratory Practices were published, they appear to have been conducted using appropriate methods and to have been fully reported.

(4) (5) (6)

5.1.3 ACUTE DERMAL TOXICITY

Type : LD_{50}
Value : > 5 ml/kg bw
Species : Rabbit
Strain : New Zealand white
Sex : Male/female
Number of animals : 8
Vehicle : Undiluted
Year : 1980
GLP : No data
Test substance : Diesel fuel, sample API 79-6 (See section 1.1.1.)

Method : 24 hours prior to testing 5 male and 5 female rabbits were weighed, and their dorsal skin shaved. The shaved skin of 2 males and 2 females was abraded with a hypodermic needle, sufficient to cut the stratum corneum but not so deep as to disturb the dermis or cause bleeding. A single 5 ml/kg dose (calculated on day 0 body weights) of test material was applied to a gauze sponge which was then placed on the skin test site. The gauze was covered by an occlusive dressing.
After a 24 hour exposure period the dressings were removed and any residual test material was removed from the skin by wiping with gauze sponges. Throughout the study the animals were examined for behavioral reactions and other signs of toxicity and the skin test sites were examined for local reactions. Body weights were recorded at the beginning of the study and again after 7 and 14 days. Surviving animals were sacrificed on the 14th day and were subjected to a gross necropsy.

Result : There were no clinical signs of toxicity and the animals did not lose weight during the study.
Erythema followed by drying and flaking of the skin was noted at the test site in all rabbits. At gross necropsy congested kidneys were noted in 4 rabbits, two animals had hemorrhages in the trachea and one rabbit had a congested liver.

Conclusion : The acute dermal LD_{50} of the test material was greater than 5 ml/kg. Exposure to the test material did not cause any compound-related changes.

Reliability : (1) valid without restriction

Although the study was conducted before Good Laboratory Practices were published, the study appears to have been conducted using appropriate methods and to have been fully reported.

(7)

Type : LD_{50}
Test substance : Home heating oil containing cracked stock (See section 1.1.1.)

Result : API reported acute dermal toxicity studies on three samples of home heating oil prepared from blends of straight run and cracked stocks. The LD_{50} values are shown below

Sample	Saturates content (%)	LD50 ml/kg	API report no.
API 78-4	67.8	>5	API 27-32068
API 78-2	73.4	>5	API 27-32771
API 78-3	79.2	>5	API 27-32773

Reliability : (1) valid without restriction
Although the studies were conducted before Good Laboratory Practices were published, they appear to have been conducted using appropriate methods and to have been fully reported.

(4) (5) (6)

5.2.1 SKIN IRRITATION

Species : Rabbit
Concentration : Undiluted
Exposure : Occlusive
Exposure time : 24 hour(s)
Number of animals : 6
PDII : 6.81
Method : Draize Test
Year : 1980
GLP : No data
Test substance : Diesel fuel, sample API 79-6 (See section 1.1.1.)

Method : Four test sites approximately one inch square were prepared on each of 3 male and 3 female rabbits by clipping. Two of the sites on each animal were abraded with a hypodermic needle, sufficient to cut the stratum corneum, but not deep enough to either disturb the dermis or cause bleeding. For each test site, 0.5 ml of test material was applied to a gauze patch which was then placed on the skin. The gauze patches were secured in place with a bandage and an occlusive covering.
24 hours after the patches were applied they were removed and any surplus test material was removed from the skin by wiping with a gauze sponge.
24 hours after patch removal the skin at the test site was examined and scored for erythema and edema (Draize scale).

Result : At 24 hours the skin of all rabbits was very irritated. Blisters had formed and some of these had opened.
At 72 hours the sites were still very irritated and open sores were observed in 2 rabbits.
At day 7 scabs had formed at most test sites
At day 14 the skin was healing but was still dry and flaky and there had been no hair growth.

Average scores for erythema and edema are listed below.

	Exposure time (Hrs)	Average score
Erythema		
Intact skin	24	4.0
	72	3.83
Abraded skin	24	4.0
	72	3.83
Edema		
Intact skin	24	3.0
	72	2.92
Abraded skin	24	3.0
	72	2.67

Conclusion : Extremely irritating
Reliability : (1) valid without restriction
Although the study was conducted before Good Laboratory Practices were published, the study appears to have been conducted using appropriate methods and to have been fully reported.

(7)

Species : Rabbit
Test substance : Home heating oil containing cracked stocks (See section 1.1.1.)

Result : API reported skin irritation studies on three samples of home heating oil prepared from blends of straight run and cracked stocks.
The primary irritation indices are shown below

Sample	Saturates content (%)	Irritation index	API report no.
API 78-4	67.8	3.83	27-32068
API 78-2	73.4	3.37	27-32771
API 78-3	79.2	3.98	27-32773

Reliability : (1) valid without restriction
Although the studies were conducted before Good Laboratory Practices were published, they appear to have been conducted using appropriate methods and to have been fully reported.

(4) (5) (6)

5.2.2 EYE IRRITATION

Species : Rabbit
Concentration : Undiluted
Dose : 0.1 ml
Comment : Rinsed after (see exposure time)
Number of animals : 9
Result : Not irritating
Method : Draize Test
Year : 1980
GLP : No data
Test substance : Diesel fuel, sample API 79-6 (See section 1.1.1.)

Method : Four male and 5 female rabbits were used in this study.
0.1 ml of undiluted test material was placed on the everted lower eyelid of the right eye of each rabbit. The left eyes were not treated and served as controls. 30 seconds after application, the eyes of 3 rabbits (2 females, one male) were flushed for one minute with warm distilled water.

Scoring for ocular lesions was done at 24, 48 and 72 hours after treatment using the Draize scale. Fluorescein was used to aid evaluation at the 24 hour reading.

Result : All rabbits scored zero at every observation period.
Those rabbits that had their eyes rinsed appeared no different to the unrinsed group.

Reliability : (2) valid with restrictions
Although the study was conducted before Good Laboratory Practices were published, the study appears to have been conducted using appropriate methods and to have been fully reported.

(7)

Species : Rabbit

Result : API reported eye irritation studies on three samples of home heating oil prepared from blends of straight run and cracked stocks.
The primary irritation indices are shown below

Sample	Saturates content (%)	Eye Irritation Index rinsed eye	Unrinsed eye	API Report No.
API 78-4	67.8	0	0	27-32068
API 78-2	73.4	0.7	0.7	27-32771
API 78-3	79.2	0	1.33	27-32773

Reliability : (1) valid without restriction
Although the studies were conducted before Good Laboratory Practices were published, they appear to have been conducted using appropriate methods and to have been fully reported.

(4) (5) (6)

5.3 SENSITIZATION

Type : Patch-Test
Species : guinea pig
Concentration : 1st: Induction undiluted occlusive epicutaneous
2nd: Challenge undiluted occlusive epicutaneous
Number of animals : 10
Result : Not sensitizing
Year : 1980
GLP : No data
Test substance : Diesel fuel, sample API 79-6 (See section 1.1.1.)

Method : 0.5 ml of undiluted test material was applied to a one inch square gauze patch which was then placed on the shorn, depilated skin of 10 male guinea pigs. The applied material was covered with an occlusive dressing which was left in place for 6 hours. This procedure was repeated at the same test site, three times a week for 3 weeks. After the tenth application, the animals were allowed to rest for two weeks without any treatment. After the two weeks rest period 0.5 ml of the test material was applied to the shorn skin of each animal, but on this occasion to a virgin site on the animal's other side.

This procedure was also followed with a group of 10 positive control animals in which chlorodinitrobenzene (0.05% in ethanol) was applied.

24 hours after each application of test or control material the treated site was evaluated and scored for erythema and

Result : The average scores for erythema and edema during the induction phase and after challenge are tabulated below.

	Test material	Positive control
Induction		
Erythema	1.3	1.3
Edema	0.3	0.3
Challenge		
Erythema	1.3	1.9
Edema	0.3	0.7

The authors concluded that the test material was not sensitizing since the differences between induction and challenge scores were not statistically significant.

Reliability : (2) valid with restrictions
The response to the positive control challenge was not remarkable. It is doubtful therefore, that the assay used is sufficiently sensitive.
Although the study was conducted before Good Laboratory Practices were published, the study appears to have been conducted using appropriate methods and to have been fully reported.

(7)

5.4 REPEATED DOSE TOXICITY

Type :
Species : Rat
Sex : Male/female
Strain : Sprague-Dawley
Route of admin. : Dermal
Exposure period : Four weeks
Frequency of treatm. : Daily, five days per week for four weeks
Doses : 0.5, 2 & 5 ml/kg/day
Control group : Yes
Year : 1986
GLP : Yes
Test substance : Diesel fuel No. 2
Sample F-75-01 is a diesel fuel No. 2
Its composition is:
Saturates 60.4%
Aromatics 39.6%

Method : Three groups of ten male and ten female young adult Sprague-Dawley rats were administered test material to the shorn dorsal skin once daily, five days per week for four weeks at doses of 0.5, 2 or 5 ml/kg/day.
The applied material was covered with an occlusive patch for six hours.
A further group of ten male and ten females served as sham-treated controls.
The animals were observed twice daily for clinical signs of toxicity. Dermal irritation at the application site was assessed daily prior to the next application of test material. An assessment of dermal irritation was also made 24 hours after the final application, just prior to necropsy.
Body weights were recorded three times weekly and just prior to necropsy.
At necropsy, a blood sample was taken for the following hematological and

clinical chemical determinations:

Hematology	Clinical chemistry
Erythrocyte count	Glucose
Total leukocyte count	Blood urea nitrogen
Differential leukocyte count	Alkaline phosphatase
Hemoglobin	SGOT
Hematocrit	SGPT
	Total protein

The following organs were weighed:
Liver
Kidneys (both)
Testes (both)/Ovaries (both)
Brain
Spleen

The following tissues were taken, were fixed and prepared for microscopic examination.
Spleen
Liver
Kidneys (both)
testes/ovaries
Brain (cerebrum, cerebellum, pons)
Skin (treated and untreated)
Bone marrow (smear)
Gross lesions.
The following tissues were removed and preserved but were not examined.

Salivary glands	Thyroid glands
Thymus	Adrenal glands
Trachea	Stomach
Esophagus	Duodenum
Cervical lymph node	Jejunum
Heart	Ileum
Colon	Lungs

Histopathology was done on the sham treated control group and the high dose group animals only.

Statistical methods
Body weights, clinical chemistry, terminal body weights, absolute and relative organ weights were examined using Dunnet's t-test at the 5% probability level.

Result **:** There were no mortalities or any other treatment-related clinical signs of toxicity during the study with the exception of an effect on body weights and the occurrence of skin irritation.

After the second week of the study, the body weights of the mid and high dose males were less than those of the controls and this difference persisted throughout the study. At the end of the study the weight gains of the mid and high dose males were 43 and 13% respectively of those of the controls.

Skin irritation occurred at all dose levels as follows:
Low dose (0.5 ml/kg/day)
Moderate erythema with slight edema which tended to be progressive over the first two weeks. The irritation plateaued after the second week. After the weekends, when there was no dosing, the skin showed some recovery.

Eschar and fissuring peaked during the second week and declined in intensity over the last two weeks of the study.

Mid dose (2 ml/kg/day)
Severe irritation which was progressive during the first week. The level of irritation plateaued during the second week and remained constant thereafter. Eschar and fissuring were evident during the first week and became progressively worse with time. There was evidence of recovery during the fourth week.

High dose (5 ml/kg/day)
Severe irritation which progressively worsened during the first week but plateaued during the second week. The lesions included moderate erythema, mild edema including ulceration, eschar and fissuring. There was some evidence of recovery during the last week of the study in that the degree of irritation appeared to diminish in intensity.

There were some differences in organ/body weight ratios recorded but these were not considered to be a direct effect of treatment but rather as a consequence of reduced body weights of the animals.

Although there were some differences in some clinical chemistry and hematological parameters, they were not dose-related and were not, therefore, considered to be treatment-related.

At gross necropsy, the only treatment-related finding was the presence of skin irritation which included dry skin and scab formation and was consistent with the clinical findings throughout the study.

Histological examination of the tissues taken from rat in the high dose group did not reveal any treatment-related effects other than skin irritation at the site of application. These included acanthosis, hyperkeratosis, dermal fibrosis, epidermal crusting, dermal inflammation and ulceration.

In conclusion the primary effect of dermal exposure to test material was skin irritation at all dose levels, with more irritation occurring at the highest two dose levels.
In the highest two dose groups body weight gains were also reduced.
There were no other biologically significant findings.

Reliability : (1) valid without restriction

(38)

Type	:	Sub-acute
Species	:	Rabbit
Sex	:	Male/female
Strain	:	New Zealand white
Route of admin.	:	Dermal
Exposure period	:	3 weeks
Frequency of treatm.	:	5 days each week for 3 weeks
Doses	:	0.2, 0.67 & 2.0 g/kg
Control group	:	Yes
Year	:	1984
GLP	:	Yes
Test substance	:	Diesel fuel LF-7765 RI

Method : The test material was applied undiluted to the shorn skin of groups of 10

male and 10 female rabbits at dose levels of 0.2, 0.67 and 2.0 g/kg/day. Dose was adjusted by altering the dose volume. The treated skin site was not covered after application of test material and ingestion was prevented by fitting collars to the animals. Two groups of 10 male and 10 females served as untreated controls and sham-treated controls. Animals were dosed once each day, 5 days each week for 3 weeks. All rabbits were observed twice daily for morbidity and mortality except on non-dosing days when the observations were made only once per day. Dead rabbits were removed for necropsy and animals not expected to survive were sacrificed and necropsied. Body weights were recorded 24 hours prior to the study, weekly during the study and immediately prior to sacrifice at termination. The skin at the application site was examined 24 hours after the first application of test material and weekly thereafter. The skin reactions were scored according the Draize scale.
Blood samples were obtained by cardiac puncture from each rabbit (after 18-24 hours fasting), and from each surviving animal at the end of the study. The blood samples were analyzed for a range of clinical chemical and hematological parameters.
All rabbits whether dying or killed were subjected to a post mortem examination. Major organs were weighed and a wide range of tissues preserved for histopathological examination.

Result : 2/10 females in the highest dose group died and one male at this dose level was sacrificed in extremis.
Clinical signs, when observed, were related to the degree of skin irritation that occurred during the study. There were signs of severe dermal irritation at the treated site consisting of scaling, scabbing and eschar formation. This severe irritation was seen in all rabbits treated with the test material. Although only minimal irritation occurred after the first exposure to the test material the severity increased as treatment progressed. In all treatment groups hyperirritability and hair loss was observed. In the highest dose group hind limb paresis and decreased motor activity were also observed. This was attributed to the degree of skin irritation since the affected skin became cracked with dense scabbing. The skin in these areas became tight and unpliable resulting in painful movement for the animals.
Body weight gains were lower than controls for the two highest dose groups. The low dose group had reduced body weight gains only in the third week of the study.
The following clinical chemical and hematological differences with controls were also observed:

	2 g/kg	0.67 g/kg	0.2 g/kg
SGOT	increased	-	-
Globulin	increased	-	-
Potassium	increased	-	-
Albumin/globulin ratio	decreased	-	-
Albumin	decreased	-	-
Alkaline phosphatase	decreased	decreased	-
Glucose	increased	increased	-
WBC	increased	increased	-
RBC	decreased	-	-
Hemoglobin	decreased	-	decreased
Hematocrit	decreased	-	-
MCHC	decreased	decreased	decreased
Differential WBC			
Mature Neutrophils	increased	-	-
Lymphocytes	decreased	-	-
Basophils	decreased	-	-

There were no significant differences in either male or female organ or organ to body weight ratios in any dose group when compared to the untreated controls. However when the data from both sexes were combined and these were compared to control values significant differences were found. These were as follows:

	Dose (g/kg)		
	2	**0.67**	**0.2**
Brain (absolute wt.)	low	low	-
Brain (relative wt.)	high	high	-
Liver (absolute wt.)	low	low	low
Liver (relative wt.)	high	-	-
Kidney (absolute wt.)	low	low	low
Kidney (relative wt.)	high	-	-
Heart (absolute wt.)	-	low	-
Heart (relative wt.)	high	-	-
Adrenals (absolute wt.)	-	-	-
Adrenals (relative wt.)	high	-	-

At gross necropsy skin changes were observed at all dose levels. These included thickened, scaly, flaky, cracked and crusty skin at the application site. Essentially all of the treated rabbits had enlarged prefemoral lymph nodes, some red in color. Several of the high dose rabbits also had enlarged axillary and inguinal lymph nodes. Stomach ulceration and thickened mucosa in some rabbits and small testes and ovaries in a few of the high and mid-dose animals was attributed to the stress of test article application or collar placement.

Histopathological examination revealed epidermal hyperplasia, hyperkeratosis, parakeratosis, dermatitis, necrosis and ulceration at the application site. The incidence was not dose-related but the severity was. There was a treatment-related enlargement of prefemoral lymph nodes and were due to a proliferation of lymphocytic and reticuloendothelial cells. Testicular tubular epithelial degeneration was observed in all study groups but there was a higher incidence in the groups exposed to the test material. This change together with giant cell formation was only observed in males in the high dose group.

Test substance : SAMPLE LF-7765 RI was described as a pink liquid with a viscosity similar to that of gasoline.

Reliability : (1) valid without restriction
Although it is not clear whether the study was carried out according to Good Laboratory Practices, it was subjected to a quality assurance assessment and appears to have been conducted using appropriate methods and to have been fully reported.

(26)

Species : Rabbit
Sex : Male/female
Strain : New Zealand white
Route of admin. : Dermal
Exposure period : 24 hours
Frequency of treatm. : Daily for 5 days, two days rest and daily for further 5 days
Doses : 1, 3 & 10 ml/kg
Control group : Yes, concurrent no treatment
Year : 1979
GLP : Yes
Test substance : Diesel fuel, sample API 78-4 (predominantly saturates) See section 1.1.1.

Method : Undiluted test material was applied, using a four-inch square gauze, to the shorn skin of groups of 4 male and 4 female adult New Zealand White rabbits. Dose groups were 1, 3 and 10 ml/kg. An untreated group of 4 males and 4 females served as controls.
The applied gauze was covered with an occlusive dressing and left in place for 24 hours. After 24 hours it was removed and a fresh dose applied. This dosing regime was continued for 5 consecutive days, followed by two days rest and then with 5 consecutive days of further dosing.
Animals were observed for mortality, local reactions and behavioral changes. Initial and final body weights were recorded. Any animals that died during the study as well as those killed at termination were subject to necropsy. All significant gross pathological alterations were recorded.
Skin from the test site, liver, kidney, spleen and urinary bladder were submitted for histopathologic examination.

Remark : This study only demonstrated the irritant nature of the test material when applied repeatedly under occlusion to the skin. Any other changes were probably secondary to the severe skin irritation that occurred.

Result : Mortality and weight change in the respective groups was as follows:

Dose group (ml/kg/day)	Weight change (kg)	Mortality (%)
0	0.2 gain	0
1	0.08 gain	0
3	0.2 loss	25
10	0.7 loss	87.5

The most significant daily observation recorded at all three dose levels was the progressive deterioration of the skin at the treated site. It became thickened and necrotic and the animals were distressed by the treatment.

At gross post mortem, the 10 ml/kg animals were anorexic and had severe skin lesions. Treatment-related lesions were seen in animals at all dose groups.
The only significant histological findings were those associated with the severe skin lesions.

Reliability : (1) valid without restriction

(6)

Species : Rabbit
Strain : New Zealand white
Route of admin. : Dermal

Result : Four samples of distillate fuels have been tested in two week repeat dose toxicity studies. The results are summarized in the following table and, for completeness, these include the data from the preceding robust summary.

Sample	Dose (ml/kg/day)	Growth rate/ mortality	API Report No.
Home heating oils			
78-3	2.5	no effect)	
	4	no effect)	27-32773
	10	8/8 died, weight loss)	
78-2	1	no effect)	

	2.5	1/8 died)	27-32771
	10	6/8 died, weight loss)	
78-4	1	no effect)	
	3	2/8 died, weight loss)	27-32068
	10	7/8 died, weight loss)	

In all the studies, skin irritation was severe at all dose levels.

Diesel fuel			
79-6	4 ml/kg/day	no effect)	27-32817
	8 ml/kg/day	67% mortality)	

(4) (5) (6) (7)

5.5 GENETIC TOXICITY 'IN VITRO'

Type : Ames test
System of testing : S. Typhimurium Strain TA 98
Test concentration : 1 to 60 µl
Metabolic activation : With and without
Result : Positive
Method : Modified Ames Assay
Year : 1991
GLP : No data
Test substance : 3 Samples of diesel fuel (See section 1.1.1. sample Nos. 22, 23 & 24)

Method : A modified Salmonella mutagenicity assay was performed at the Mobil Environmental and Health Science Laboratory. The technique that was used has been described elsewhere (Blackburn et al 1984 & 1986).

The middle distillate samples (2 ml) were dissolved in cyclohexane and the solution was then extracted with DMSO (10 ml). These extracts were tested in Salmonella typhimurium strain TA98.
The concentrations of DMSO extract used were: 60, 50, 40, 30, 20 15, 10 and 5 µl/60 µl. Extra concentrations were used for some assays.
Positive controls were 2.0 µg 2-aminoanthracene, 10.0 µg benzo(a)pyrene and 25 µg 2-nitrofluorene in 50 µl DMSO per bacterial plate.
Metabolic activation was accomplished by using an eight- fold higher concentration of the liver S9 fraction obtained from Arachlor-induced Syrian Hamsters rather than rats.
NADP cofactor was also increased from the normal 4 to 8 mM.

Result : A mutagenicity index (MI) was calculated which represented the slope of the dose response curve for each of the samples.
Previous studies have established that materials with an MI of less than or equal to 1.0 have not been associated with a tumorigenic response in skin painting bioassays, whereas those with MIs greater than 1.0 have been associated with a tumorigenic response.
The MIs for the middle distillate samples were:

Sample	**Mutagenicity Index**
22	1.7
23	3.9
24	2.0

The authors of the report concluded that the three samples of diesel fuel were mutagenic.

(10) (11) (15)

Type : Ames test

Result : Diesel fuel was evaluated in plate and suspension assays using S.typhimurium strains TA 1535, 1537, 1538, 98 and 100 and in yeast S. cerevisiae D4.
The assays were conducted without and with rat liver S9 activation.
The diesel fuel was negative in these assays.

(1)

Type : Mouse lymphoma assay
System of testing : Forward mutation assay using cell line L5178Y TK+/-
Test concentration : 0.125 to 0.5 µl/ml without activation and 0.064 to 0.5 µl/ml with activation.
Metabolic activation : With and without
Result : Negative
Year : 1978
GLP : No data
Test substance : No.2-DA (See section 1.1.1.)

Method : The test material was dissolved in ethanol for this assay.
Two positive control substances were used viz Ethyl methane sulphonate (EMS) at a concentration of 0.5 µl/ml and Dimethylnitrosamine (DMN) at a concentration 5.0 µl/ml.
Doses of test material used in the assay were based on the results of a cytotoxicity study carried out prior to the mutagenicity assay.

For the mutation assay the lymphoma cells were exposed for 4 hours to test material at dose levels ranging from 0.125 to 0.50 µl/ml without activation and 0.064 to 0.5 µl/ml with S-9 activation. After exposure to the test material, the cells were allowed to recover for 3 days. Mutants were detected by cloning the cells in the selection medium for 10 days.
Surviving cell populations were determined by plating.
A mutant frequency was derived by dividing the number of clones formed in the BUdR-containing selection medium by the number found in the same medium without BUdR.

A compound is considered mutagenic if:

a. A dose response relationship is observed over three of the four dose levels used
b. The minimum increase at the high level of the dose response curve is at least 2.5 times greater than the solvent control.
c. The solvent control data are within the normal range of the spontaneous background for the TK locus.

Result : The mutant frequencies and percentage relative growth are summarized in the following table.

Non-activation Group	**Mutant frequency**	**% Relative growth**
Solvent control	0.0231	100
Negative control	0.2143	126.1
EMS control	0.9758	37
Test material		
0.125 (µl/ml)	0.0658	59.0
0.188 (µl/ml)	0.085	44.5
0.25 (µl/ml)	0.3469	12.3

0.375 (µl/ml)	0.1264	17.3
0.5 (µl/ml)	0.0396	9.3
With activation		
Solvent control	0.0204	100
Negative control	0.0321	92.9
DMN control	0.9365	50.3
Test material		
0.064 (µl/ml)	0.0105	93.7
0.125 (µl/ml)	0.0129	98.5
0.188 (µl/ml)	-	22.7
0.25 (µl/ml)	0.0870	5.7
0.375 (µl/ml)	0.0183	2.1
0.5 (µl/ml)	0.1235	2.0

(1)

Type : Mouse lymphoma assay
Test substance : Sample API 78-4. See section 1.1.1.

Result : Home heating oil (API Sample 78-4, containing 50% cracked stock) was positive with and without S9 activation. (Ref API 27-30140)

(2)

5.6 GENETIC TOXICITY 'IN VIVO'

Type : Cytogenetic assay
Species : Rat
Sex : Male
Strain : No data
Route of admin. : i.p.
Exposure period : Up to 48 hours and 5 days
Doses : 0.6, 2.0 & 6.0 cc/kg
Year : 1978
GLP : No data
Test substance : No.2-DA (See section 1.1.1.)

Method : Acute and subchronic studies were carried out. The design of the two studies is summarized in the following table.
In the acute study a single intraperitoneal dose was given to the animals which were then sacrificed at the time intervals shown. Two hours prior to being killed, colchicine was given as a single dose (4 mg/kg) to arrest dividing cells in metaphase.
Acute study

Treatment	**No. of animals killed hrs after dosing**		
	6	**24**	**48**
0.6 cc/kg	5	5	5
2.0 cc/kg	5	5	5
6.0 cc/kg	5	5	5
TEM positive control	5	5	5
Solvent control	5	5	5

In the subchronic study a group of 5 males at each dose level (same doses as for acute study) was given a single intraperitoneal dose of test material once every 24 hours for 5 days. Animals in this study were killed 6 hours

after the last dose was given. Cells were arrested in metaphase by the use of colchicine at the same level as that used in the acute study.

The positive control material was triethylene melamine (TEM), used at a dose of 0.5 mg/kg. The solvent control was 0.85% aqueous saline.

Bone marrow was aspirated from the canals of the femurs and tibias of the lower limbs. The cells from the bone marrow plug were washed, fixed and spread on slides and stained for examination. 50 spreads were located for each animal and when of suitable quality, the chromosomes were counted and evaluated for abnormalities.

Result : Similar aberrations were observed throughout the treatment and negative control groups, the only difference being the frequency of aberration.
The results of the evaluation are summarized in the following table.

Group	Time (hrs)	No of cells	Cells with 1 or more aberrations	No. animals without aberrations*	MI**
Saline	6	53	0 (0%)	2 (2)	0.7
	24	225	6 (2.6%)	2 (5)	3.2
	48	200	1 (0.5%)	3 (4)	3.6
	5 doses	250	2 (0.8%)	4 (5)	10.6
TEM	24	144	68 (47.2%)	0 (5)	1.4
Diesel 0.6 ml/kg	6	189	5 (2.6%)	0 (5)	5.9
	24	48	1 (2.1%)	4 (5)	1.7
	48	186	1 (0.5%)	4 (5)	2.3
	5 doses	250	4 (1.6%)	2 (5)	3.6
Diesel 2.0 ml/kg	6	228	8 (3.5%)	1 (5)	6.3
	24	86	5 (5.8%)	2 (5)	1.7
	48	216	2 (0.9%)	3 (5)	4.5
	5 doses	250	1 (0.4%)	4 (5)	7.4
Diesel 6 ml/kg	6	100	1 (1.0%)	1 (2)	4.0
	24	227	5 (2.2%)	2 (5)	4.7
	48	93	5 (5.4%)	2 (4)	3.8
	5 doses	200	10 (5.0%)	0 (4)	6.0

* ( ) Number in parenthesis is No. of animals examined.
** Mitotic Index

For each dose group, the total number of cells with aberrations were combined and the mean % aberrations was estimated. These data formed the basis for the report's conclusion, that at a dose level of 2 and 6 ml/kg diesel fuel was clastogenic.

Group	Total No. cells with aberrations	Mean % aberrations
Control	9	1.0
TEM	68	47.2
Diesel 0.6 ml/kg	11	1.7
Diesel 2.0 ml/kg	16	2.7
Diesel 6.0 ml/kg	21	3.4

Reliability : (2) valid with restrictions

(1)

Type : Dominant lethal assay
Species : Mouse
Sex : Male
Strain : CD-1
Route of admin. : Inhalation
Exposure period : 6 hours / day, 5 days / week for 8 weeks
Doses : 100 & 400 ppm
Result : Negative
Year : 1980
GLP : No data
Test substance : No. 2-DA (See section 1.1.1.)

Method : Groups of 12 male mice were exposed by inhalation to diesel fuel at airborne concentrations of 100 and 400 ppm. Exposures were for 6 hours a day, 5 days each week for 8 weeks (40 doses). A group of 12 male mice served as negative controls and were placed in the inhalation chambers but not exposed to diesel fuel. On day 40 of the dosing schedule a positive control group of 12 male mice were each given an acute intraperitoneal dose (0.3 mg/kg) of triethylenemelamine (TEM). Following completion of the exposure to test material the males were sequentially mated to two females per week for 2 weeks. After mating, the females were separated from the males and housed separately until killed. At the end of each 5 day mating period, the males were rested for 2 days and then mated with 2 new females.
14 days from the middle of the mating week, the females were killed and necropsied. At necropsy the uteri were examined and the number of living and dead implantations counted if present. Based on the data obtained at necropsy, the following parameters were calculated:
fertility index
total number of implantations
dead implantations
proportion of females with one or more dead implantations
proportion of females with two or more dead implantations
ratio of dead/total implants
ratio of living implants/total implants.

Result : The results were as follows:

	Dose Group			
Parameter	100 ppm	400 ppm	Negative control	Positive control
Fertility index				
Week 1	0.727	0.750	0.708	0.458
Week 2	0.955	0.792	0.833	0.792
Implants/female				
Week 1	11.06	10.72	11.18	8.36
Week 2	10.81	11.68	10.95	7.42
Resorptions/female				
Week 1	.375	.5	.77	4.73
Week 2	.71	.32	.55	4.9
Proportion of females with 1 or more dead implants				
Week 1	.32	.39	.41	1.0
Week 2	.52	.26	.55	1.0

Proportion of females with 2 or more dead implants				
Week 1	.06	.11	.24	1.0
Week 2	.14	.05	0	.95
Dead implants/Total implants				
Week 1	.03	.04	.07	.57
Week 2	.07	.03	.05	.66
Living implants/pregnant female				
Week 1	10.7	10.2	10.4	3.6
Week 2	10.1	11.4	10.4	2.5

Conclusion : The sensitivity of the assay was confirmed by the results with the positive control. The test material did not cause any significant pre- or post implantation losses when compared to the negative control.

(8)

5.7 CARCINOGENICITY

Species : Mouse
Sex : Male
Strain : C3H
Route of admin. : Dermal
Frequency of treatm. : Twice weekly for lifetime
Doses : 50 microliters
Result : Positive
Control group : Yes
Year : 1985
GLP : No data
Test substance : Diesel fuel LF-7765 RI
SAMPLE LF-7765 RI was described as a pink liquid with a viscosity similar to that of gasoline.

Method : 50 microliters of undiluted test material was applied to the shorn dorsal skin of a group of 50 male mice twice weekly for the lifetime of the animals. The applied dose was not spread mechanically or covered, but was allowed to spread of its own accord.
A group of 50 male mice served as sham-treated controls.
Animals were observed twice daily on weekdays and daily on weekends throughout the study. Any mouse considered not likely to survive until the next observation time was sacrificed and necropsied.
Body weights were recorded prior to initiation of the study, then weekly for the first four weeks, biweekly for the following 8 weeks and monthly thereafter. Animals with one or more skin masses were excluded from the body weight database to preclude skewing of the data. All mice were subjected to an extensive necropsy and a wide range of tissues were fixed for subsequent histopathological examination.

Result : Five mice died during the first nine weeks of the study. None of the deaths were considered to be treatment-related and were excluded from any subsequent statistical analysis.
The mean lifespan of the treated mice was 78.1 ± 24.2 weeks which was significantly shorter than 91.8 ± 82.5 weeks for the controls. When the data were assessed in 12 week intervals, the mortality rate of the treated mice was greater than the controls. There was a significant increase (9 vs. 0) in the incidence of malignant skin tumors (squamous cell carcinoma or fibrosarcoma) in the treated mice compared to the controls.
The mean time to appearance of histologically confirmed tumors was 94.4 ± 9.4 weeks.
The body weights and body weight gains of the treated mice were greater

than those for the controls. Unexplainably, by the end of the first year, the treated rats were on average 3 g heavier than the controls (33 vs. 3 g) and this difference remained throughout the study, the reason for which is not clear.
Seven of the 46 treated mice developed skin masses compared to zero out of 49 in the control group. The first of the masses was recorded during the 84th week. Other lesions of the treated skin included sloughing of the skin and lesions resembling infection both of which were seen more frequently in the treated animals. Increased motor activity lasting for one to two hours was a consistent observation in the treated mice but only seen in one of the sham-treated controls. Four treated mice were observed with rectal prolapse but this was not seen in the control animals. Some age-related changes were observed in both treated and control animals but hair loss, distended abdomens, abdominal masses and distended penises was observed more frequently in the treated animals than in the controls.
At necropsy none of the control mice had skin masses while masses were observed in seven of the treated mice.
Lesions observed in both groups but more frequently in the treated animals are shown below:

	Treated	**Control**
Final group size	46	49
Sloughing skin at the application site	41	1
Lesions resembling infection of the skin	18	2
Inguinal lymph node enlargement	29	16
Enlargement of the spleen	10	5
Distension of urinary bladder	8	1
Presence of calculi in the urinary bladder	4	0
Masses/nodules of the kidneys	5	0

All other observations at necropsy occurred in both treated and control animals at a similar incidence.

The only tissues that were processed for histopathology were the treated skin from all mice and all tissues considered to be outside normal limits at necropsy.
With the exception of those listed below, all lesions observed during microscopic examination were considered to be spontaneous and common for ageing mice of the strain.

	Treated	**Control**
Final group size	46	49
ORGAN/LESION		
Skin		
Squamous cell carcinoma	8/46	0/49
Fibrosarcoma	1/46	0/49
Acanthosis	33/46	1/49
Hyperplasia	1/46	0/49
Hyperkeratosis	2/46	0/49
Dermis - fibrosis/fibroplasias	26/46	0/49
Necrosis	8/46	1/49
Inflammation	3/46	0/49
Ulceration	1/46	0/49
Epidermal inclusion cyst	1/46	0/49
Accumulation of pigment	6/46	0/49
Dermatitis	1/46	0/49
Non-treated skin		
Squamous cell carcinoma	1/46	0/49

Liver*		
Hepatocellular carcinoma	22/29	25/36
Hepatocellular adenoma	4/29	4/36
Lungs*		
Bronchogenic carcinoma	0/7	2/17
Alveologenic carcinoma	1/7	1/17
Alveologenic adenoma	2/7	1/17
Hematopoietic lymphoreticular system*		
Malignant lymphoma	0	1/35
Pancreas+		
Islet cell adenoma	0	1/1

* Incidence of lesions in these organs based on number exhibiting gross abnormalities at necropsy.

Test substance : SAMPLE LF-7765 RI was described as a pink liquid with a viscosity similar to that of gasoline.

(27)

5.8.2 DEVELOPMENTAL TOXICITY/TERATOGENICITY

Species : Rat
Sex : Female
Strain : Sprague-Dawley
Route of admin. : Inhalation
Exposure period : Days 6 through 15 of gestation
Doses : 100 and 400 ppm
Control group : Yes
NOAEL maternal tox. : 401.5 ppm
NOAEL teratogen. : 401.5 ppm
Year : 1979
GLP : No data
Test substance : No.2-DA (See section 1.1.1.)

Method : Groups of 10 presumed-pregnant female rats (224-230 g) were exposed in chambers to diesel fuel at nominal atmospheric concentrations of 100 and 400 ppm. A further group of presumed-pregnant females served as controls. Exposures were for 6 hours each day from day 6 through day 15 of gestation.
Food and water were provided ad libitum except during the exposure periods.
The rats were weighed on days 0, 6, 15 and 20 of gestation.
Food intakes were assessed for the periods days 0-6, 7-15 and 16-20.
There were daily observations for general appearance, behavior and condition. On day 20 of gestation the rats were killed and the visceral and thoracic organs were examined. The uterus was removed and opened and a record made of the number of implantation sites and their placement in the uterine horns, the number of fetuses (alive and dead), and the number of resorption sites.
Fetuses were removed and weighed and were examined externally for abnormalities. One third of the fetuses were fixed for subsequent examination for changes in the soft tissues of the head, thorax and visceral organs. The remaining fetuses were examined for skeletal abnormalities after staining with Alizarin Red S. The uterus and ovaries were preserved in fixative for possible future examination.

Statistical analysis
Analysis of the data was performed using the litter as a basic sampling unit. Dunnet's t-test was used to determine statistical significance ($p<0.05$) for differences between means with near normal distributions (body weights, food consumption of dams, mean pup weight based on litter averages). Ratios (nidation index and live fetuses/implantation sites ratio) were analyzed with a 2 x 2 contingency table with Yate's correction. A Wilcoxon Rank Sum was used for discontinuous parameters (e.g. number of abnormal fetuses within a litter).

Result : After analysis of samples collected during the exposure the mean chamber concentrations of diesel fuel were found to be 0, 101.8 (± 2.4) and 401.5 (± 15.39) ppm.
There were no deaths during the study and all animals were normal in appearance throughout. At necropsy two 400 ppm animals had dark mottled lungs and one 100 ppm animal had two pups with a common placenta. Neither of these observations are considered to be treatment-related.
Body weights were not affected by exposure to the test material. Food consumption of treated and control animals was similar at the time periods examined except for the 400 ppm group which had a reduced food intake during the gestation period, days 7-15 only.
No treatment-related changes were observed on observation of the uterine contents.

There was no difference in sex ratios attributable to exposure to test material. The actual numbers were:

Exposure	Males	Females
0 ppm	29	32
101.8 ppm	35	37
401.5 ppm	35	25

No treatment-related differences were found in any of the following parameters:
Nidation index (females with implantations/bred)
Females dying prior to cesarean section
Live litters
Implantation sites (for each uterine horn)
Resorptions
Litters with resorptions
Dead fetuses
Litters with dead fetuses
Live fetuses/implantation site
Mean live litter size
Average fetal weight

Examination of the offspring at delivery revealed no visible abnormalities except for subcutaneous hematomas which occurred as follows:

0 ppm group	1
101.8 ppm group	4
401.5 ppm group	4

There were no abnormalities found in the soft tissues of the fetuses after examination of the Bouin's fixed specimens.

Some changes were observed during the skeletal examination of the stained fetuses. The "unusual changes" shown in the following table consisted of retarded bone ossification and were not malformations as

such. It was noted in the report that such changes, although not normal, were frequently observed in 20 day old fetuses of the strain and source of rat used in the study. The report concluded that neither the frequency nor the character of the changes indicated an adverse effect on fetal growth and development or a teratogenic potential.

Exposure (ppm)	Fetuses examined	Fetuses normal	Fetuses with commonly found changes	Fetuses with unusual skeletal variations
0	127 (16)	70 (16)	50 (14)	7 (3)
101.8	155 (19)	68 (16)	70 (18)	17 (8)
401.5	128 (16)	66 (16)	62 (15)	0 (0)

Figures in parenthesis indicate No. of litters

Reliability : (2) valid with restrictions
The study was subjected to a Quality Assurance inspection but there is no information regarding compliance with GLP requirements. Nevertheless the study was well conducted and reported and is considered reliable.

(3)

5.9 SPECIFIC INVESTIGATIONS

Endpoint : Initiation/promotion assay
Species : Mouse
Sex : Male
Strain : CD-1
Route of admin. : Dermal
No. of animals : 30
Vehicle : Undiluted
Control group : Yes
Year : 1993
GLP : Yes
Test substance : Diesel fuel, DGMK Sample No. 22 (See section 1.1.1.)

Method : A sample of a diesel fuel was investigated in this assay.

50 µl of test material was applied undiluted to the shorn dorsal skin of 30 male CD-1 mice for 5 consecutive days of the first week of the initiating period.

Promoter: TPA (12-0-tetradecanoylphorbol-13-acetate),applied (5 µg/animal, dissolved in 50 µl acetone) twice a week from week 4 to week 28.

Initiator DMBA (7,12-dimethylbenz(a)anthracene) applied once (50 µg/animal, dissolved in 50 µl acetone) on the first day of the initiating period.

The treatment groups were as follows:

Group	Treatment
0	Acetone/TPA
1 negative control	DMBA/Acetone
2 negative control	DMBA/TPA
Test for initiating activity	
14	Diesel fuel/TPA
Test for promoting activity	
15	Diesel fuel/DMBA

The treatment regimes were:
For assessing tumor initiating potential, the test material was applied to the mice for 5 consecutive days during the first week of the initiating period.
TPA was applied twice a week from week 4 to 28.
Body weights were determined once weekly and once each week a detailed examination of the skin was performed.
At the end of the study, the animals were assessed grossly followed by histopathology of the skin and all macroscopic lesions.

Assessment of promoting activity
The animals were initiated with DMBA once on the first day of the administration. From week 4 to 28 the test material was applied to the mice twice a week.

During weeks 2 and 3 of the study, i.e. between initiation and promotion, the animals were untreated to allow a regression of possible skin alteration.

Result **:** The diesel fuel sample did not cause any body weight changes compared to controls.
Survival of the animals was also unaffected by exposure to the diesel fuel sample.
During the initiation phase, the diesel fuel sample caused slight skin irritation consisting of reddening, scale formation, and/or erosions in four of the 30 animals.
During the two week recovery period between the initiation and promotion phase the skin changes were found to be reversible.

Similar skin changes also occurred during the promotion phase.

The number of animals with neoplastic findings in the treated skin at the end of the study was follows:

Test group	**treatment**	**No. of animals with squamous cell papilloma**
0	Acetone/TPA	0
1	DMBA/Acetone	0
2	DMBA/TPA	30
14	Diesel fuel/TPA	0
15	DMBA/Diesel fuel	1

The authors concluded that the diesel fuel sample may be a promoter.

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